(54) Title: HETERO-ASSOCIATING COILED-COIL PEPTIDES

(57) Abstract: The present invention relates to methods for the identification of novel hetero-associating coiled-coil peptides and uses of these peptides for hetero-dimerization of fusion proteins. It furthermore relates to vectors, host cells useful for the production of these novel hetero-association peptides and (poly)peptides/proteins comprising these peptides.

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clone (occurrence)	LibraryA									LibraryB								
	e1	g1	e2	g2	a3	e3	g3	e4	g4	e'1	g'1	e'2	g'2	a3'	e'3	g'3	e'4	g'4
#1 (22x)	E	K	R	Q	N	K	R	R	Q	Q	E	Q	E	N	K	K	R	K
#2 (3x)	Q	K	R	R	N	K	E	E	K	E	E	K	Q	N	K	K	Q	Q
#3 (2x)	E	Q	Q	R	N	K	K	K	K	R	K	Q	E	N	E	E	Q	R
#4 (1x)	E	R	R	Q	N	K	K	E	Q	E	E	E	Q	N	Q	R	E	R
#5 (1x)	E	Q	E	E	N	K	K	R	R	K	K	K	K	N	R	K	R	K
#6 (3x)	Q	Q	E	Q	N	E	E	K	Q	R	Q	Q	K	N	R	R	K	R
#7 (2x)	E	R	K	E	N	E	E	K	R	Q	E	Q	Q	N	R	Q	K	K
#8 (1x)	E	K	K	K	N	K	K	K	K	R	Q	E	Q	N	E	Q	E	E
#9 (1x)	Q	E	Q	E	N	R	E	E	E	Q	K	K	E	N	Q	K	Q	R
#10 (1x)	R	R	R	R	N	Q	K	K	R	R	E	E	E	N	R	R	R	Q

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HETERO-ASSOCIATING COILED-COIL PEPTIDES

The present invention relates to methods for the identification of novel hetero-associating coiled-coil peptides and uses of these peptides for hetero-dimerization of fusion proteins. It furthermore relates to vectors, host cells useful for the production of these novel hetero-association peptides and (poly)peptides/proteins comprising these peptides.

Increasingly, there is a need for proteins which combine two or more functions, such as binding to two different specificities, or binding and enzymatic activity, in a single structure. Typically, proteins which combine two or more functions are prepared either as fusion proteins or through chemical conjugation of the component functional domains. Both of these approaches suffer from disadvantages. Genetic "single chain" fusions suffer the disadvantages that (i) only a few (two to three) proteins can be fused (1), (ii) mutual interference between the component domains may hinder folding, and (iii) the size of the fusion protein may make it difficult to prepare. The alternative, chemical cross-linking *in vitro* following purification of independently expressed proteins, is difficult to control and invariably leads to undefined products and to a severe loss in yield of functional material.

A third approach takes advantage of using genetic fusions of functional to association domains which lead to a self-association on co-expression in appropriate host cells. To assemble at least two different fragments fused to association domains, the domains must have a tendency to form hetero-multimers. In one approach, a natural protein or protein domain was dissected and fused to protein partners to achieve hetero-association of the fusion proteins via the reconstitution of the native-like structure of the dissected protein or protein domain (WO 96/13583).

In principle, hetero-association can be achieved with complementary helices such as the hetero-dimerizing Jun and Fos zippers of the AP-1 transcription factor (2) or other helical coiled-coil structures which are involved in the oligomerization of a wide variety of

proteins. Because of their small size and structural regularity, they have also been used as artificial domains to mediate oligomerization of various proteins (3, 4). For example, the association of two separately expressed scFv antibody fragments by C-terminally fused amphipathic helices *in vivo* provides homo-dimers of antibody fragments in *E. coli* (WO93/15210; 5, 6). Coiled coils consist of two or more amphipathic helices wrapping around each other with a slight supercoil. They contain a characteristic heptad repeat $(abcdefg)_n$ with a distinct pattern of hydrophobic and hydrophilic residues (Fig. 1A, (7, 8)). The positions a and d, which form the hydrophobic interface between the helices, are usually aliphatic and a have profound effect on the oligomerization state (9, 10). The positions b, c, e, g, and f are solvent-exposed and usually polar. The positions e and g, which flank the hydrophobic core, can make interhelical interactions between g_i and e'_{i+5} residues, and thereby mediate heterospecific pairing (11-14).

As most naturally-occurring coiled coils are homodimeric, synthetic sequences have been designed to promote specific hetero oligomerization (11, 13-15). However, it was observed that designed coiled coils which behave well as synthetic peptides failed in fusion proteins expressed in *E. coli*, as they were proteolytically degraded.

Thus, the clear disadvantage of association domains based on hetero-associating helices is their pseudo-symmetry and their similar periodicity of hydrophobic and hydrophilic residues. This structural similarity resulted in a strong tendency to form homo-dimers and thus to lower significantly the yield of hetero-dimers (2, 16). Furthermore, the formation of Jun/Fos hetero-dimers is kinetically disfavoured and requires a temperature-dependent unfolding of the kinetically favoured homo-dimers, especially Jun/Jun homo-dimers (WO 93/15210; 2, 16). Because of the need for additional purification steps to separate the unwanted homo-dimers from hetero-dimers and the resulting decrease in yield, hetero-association domains based on amphipathic helices have so far not resulted in practical advantages compared to conventional chemical coupling.

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Further, it is not currently possible to predict sequences of coiled coil-forming peptides that will simultaneously have high stability and heterospecificity as well as advantageous *in-vivo* properties, such as resistance to proteases. This is crucial to practical applications of optimal interacting heterodimers for *in vivo* studies of protein oligomerization, *e.g.* the design of bispecific miniantibodies (17).

Thus, the technical problem underlying the present invention is to provide association domains based on helical coiled-coil structures which lead to hetero-association. The solution to the above technical problem is achieved by the embodiments characterized in the claims. Accordingly, the present invention provides a method which allows to identify hetero-associating (poly)peptides. The technical approach, i.e. the design of an appropriate coiled-coil library and screening by using a library-vs-library approach is neither provided nor suggested by the prior art.

Thus, the present invention relates to a method for the identification of hetero-associating (poly)peptides comprising the steps of:

  (a) providing a library A of (poly)peptides/proteins comprising (poly)peptides A_m having the general formula:

      VAQLXEXVKTLXAXZYELXSXVQRLXEXVAQL

      wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V,

  (b) providing a library B of (poly)peptides/proteins comprising (poly)peptides B_n having the general formula:

      VDELXAXVDQLXDXZYALXTXVAQLXKXVEKL

      wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V;

  (c) combining in a common medium the (poly)peptides/proteins of said libraries A and B; and

  (d) screening or selecting for a screenable or selectable property caused by the hetero-association of a (poly)peptide A_m with a (poly)peptide B_n.

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The term "(poly)peptide" relates to molecules consisting of one or more chains of multiple, i. e. two or more, amino acids linked via peptide bonds.

The term "protein" refers to (poly)peptides where at least part of the (poly)peptide has or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its (poly)peptide chain(s). This definition comprises proteins such as naturally occurring or at least partially artificial proteins, as well as fragments or domains of whole proteins, as long as these fragments or domains have a defined three-dimensional arrangement as described above.

In this context, the commonly known one-letter code for amino acid residues is used.

The term " screenable or selectable property" refers to a property which is generated in the event of a successful interaction taking place during screening or selection. Examples for screenable selectable properties include, but are not limited to, binding to a target or presentation of a target for ligand-binding, enzymatic activity, transactivation of transcription of a reporter gene such as beta-galactosidase, alkaline phosphatase or nutritional markers such as his3 and leu, or resistance genes giving resistance to an antibiotic such as ampicillin, chloramphenicol, kanamycin, zeocin, neomycin, tetracycline or streptomycin. In another embodiment, the selectable or screenable property can be restoration of phage infectivity to a filamentous phage rendered non-infectious by deletion of the N-terminal domain(s) of the geneIII protein (U.S. Patent No. 5,514,548).

In a preferred embodiment, the invention relates to a method wherein said libraries A and B are provided by providing libraries of nucleic acid sequences encoding said (poly)peptides/proteins, followed by causing or allowing the expression of said libraries of (poly)peptides/proteins.

Methods for providing libraries of nucleic acid sequences, and for causing or allowing their expression, either in vivo after transformation, transfection or transduction of appropriate host cells, using appropriate vectors, containing all elements necessary for transcription and translation such as promotor/operator elements etc., or in vitro using in

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vitro expression systems are well known to anyone of ordinary skill in the art (see e.g. Sambrook et al., 1989, Molecular Cloning: a Laboratory Manual, 2nd ed.).

Further preferred is a method wherein said common medium are host cells, each cell harbouring nucleic acid sequences encoding a (poly)peptide/protein of each of said libraries A and B.

Most preferred is a method wherein said (poly)peptides/proteins of said libraries A and B further comprise either a N- or a C-terminal fragment of the murine DHFR enzyme, and wherein said screenable or selectable property is insensitivity of the host cell to trimethoprim by reconstitution of the DHFR enzyme on hetero-association of (poly)peptides A_m and B_n.

The DHFR assay has been published (WO 98/34120; 19) and is further exemplified in the examples.

In another embodiment, the present invention relates to a hetero-associating (poly)peptide A_m taken from the list of:

WINZIPA1: VAQLEEKVKTLRAQNYELKSRVQRLREQVAQL

WINZIPA2: VAQLRERVKTLRAQNYELESEVQRLREQVAQL

WINZIPA3: VAQLQEKVKTLRARNYELKSEVQRLEEKVAQL

WINZIPA4: VAQLEEQVKTLQARNYELKSKVQRLKEKVAQL

WINZIPA5: VAQLEERVKTLRAQNYELKSKVQRLEEQVAQL

WINZIPA6: VAQLEEQVKTLEAENYELKSKVQRLRERVAQL

WINZIPA7: VAQLQEQVKTLEAQNYELESEVQRLKEQVAQL

WINZIPA8: VAQLEERVKTLKAENYELESEVQRLKERVAQL

WINZIPA9: VAQLEEKVKTLKAKNYELKSKVQRLKEKVAQL

WINZIPA10: VAQLQEEVKTLQAENYELRSEVQRLEEEVAQL

WINZIPA11: VAQLRERVKTLRARNYELQSKVQRLKERVAQL

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Furthermore, the present invention relates to a hetero-associating (poly)peptide B_n taken from the list of:

WINZIPB1: VDELQAEVDQLQDENYALKTKVAQLRKKVEKL

WINZIPB2: VDELKAEVDQLQDQNYALRTKVAQLRKEVEKL

WINZIPB3: VDELEAEVDQLKDQNYALKTKVAQLQKQVEKL

WINZIPB4: VDELRAKVDQLQDENYALETEVAQLQKRVEKL

WINZIPB5: VDELEAEVDQLEDQNYALQTRVAQLEKRVEKL

WINZIPB6: VDELKAKVDQLKDKNYALRTKVAQLRKKVEKL

WINZIPB7: VDELRAQVDQLQDKNYALRTRVAQLKKRVEKL

WINZIPB8: VDELQAEVDQLQDQNYALRTQVAQLKKKVEKL

WINZIPB9: VDELRAQVDQLEDQNYALETQVAQLEKEVEKL

WINZIPB10: VDELQAKVDQLKDENYALQTKVAQLQKRVEKL

WINZIPB11: VDELRAEVDQLEDENYALRTRVAQLRKQVEKL

Particularly preferred is the use of a hetero-associating (poly)peptide according to the present invention for the identification of optimized hetero-associating (poly)peptides in a method according to the present invention, wherein one of the hetero-associating peptide $WinZipA_m$ as listed hereinabove is used instead of library A of (poly)peptides/proteins comprising (poly)peptides A_m in step (a) above, or wherein a hetero-associating peptide WinZip B_n as listed hereinabove is used instead of library B of (poly)peptides/proteins comprising (poly)peptides B_n in step (b) avove.

In a still further preferred embodiment, the present invention relates to an optimized hetero-associating (poly)peptide obtainable by the method of the present invention.

In a most preferred embodiment, the present invention relates to a pair of hetero-associating (poly)peptides taken from the list of:

WinZipA1 and WinZipB1

WinZipA2 and WinZipB1

WinZipA1 and WinZip B2

WinZipA3 and WinZip B3

WinZipA4 and WinZip B4

WinZipA5 and WinZip B5

WinZipA6 and WinZip B6

WinZipA7 and WinZip B7

WinZipA8 and WinZip B8

WinZipA9 and WinZip B9

WinZipA10 and WinZip B10

WinZipA11 and WinZip B11

In a yet further preferred embodiment, the invention relates to a (poly)peptide/protein comprising one of the hetero-associating (poly)peptides, or an optimized hetero-associating (poly)peptide of the present invention, and a further (poly)peptide/protein.

In that context, "(poly)peptide/protein comprising one of the hetero-associating (poly)peptides, or an optimized hetero-associating (poly)peptide of the present invention, and a further (poly)peptide/protein" refers to all constructs which comprise one of the hetero-association peptides according to the present invention and additional moieties. This comprises (poly)peptides/proteins which are expressed from a contiguous nucleic acid coding sequence. Additionally, this comprises constructs where the individual components are expressed from different nucleic acid coding sequences, or where the components are produced by peptide synthesis, and where the separate components are linked by the formation of disulfide bonds or by chemical conjugation.

Still further preferred is a (poly)peptide/protein wherein said further (poly)peptide/protein is an enzyme, a toxin, a cytokine, a metal binding domain, a transcription factor, a member of the immunoglobulin superfamily, a bioactive peptide of 5 to 15 amino acid residues, a peptide hormone, a growth factor, a lectin, a lipoprotein, a peptide which is able to bind to an independent binding entity, or a functional fragment of any said further

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(poly)peptide/protein.

In a most preferred embodiment, the present invention relates to a hetero-associated (poly)peptide/protein comprising at least two (poly)peptide/proteins of the present invention, associated by hetero-association of a hetero-associating (poly)peptide A_m and a hetero-associating (poly)peptide B_n.

The term "hetero-associated (poly)peptide/protein" refers to all bispecific and/or bivalent complexes formed by taking advantage of the hetero-associating peptides according to the present invention. These include, for example, constructs where said "further (poly)peptides/proteins" are two antibody fragments directed against different specificities. Such bispecific constructs can be used to increase selectivity of antibody-based approaches in therapy of diseases where the target cells exhibit a pattern of two cell-surface markers distinct from that of non-target cells which may present one of the two markers. Furthermore, one of the antibody specificities may be directed to a target cells, whereas the second may be used to target a drug carrier moiety selectively to the target. Additionally, antibody fragments as targeting vehicles may be combined with (poly)peptides/proteins which serve as effector domains, such as enzymes or signalling molecules.

Particularly preferred is a DNA sequence encoding a hetero-associating (poly)peptide taken from the list of WINZIPA1 to WINZIPA11 and WinZipB1 to WinZipB11, or encoding an optimized hetero-associating (poly)peptide or a (poly)peptide/protein of the present invention.

Further preferred is a DNA sequence encoding a hetero-associating (poly)peptide wherein said DNA sequence hybridizes under stringent conditions to a DNA sequence encoding a hetero-associating (poly)peptide taken from the list of WinZipA1 to WinZipA11 and WinZipB1 to WinZipB11.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that at least sequences at least 65%, more preferably at least 70%, and even more preferably at least 75% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, New York. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions is hybridization in 6 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 x SSC, 0.1% SDS at 50°-65°C.

In still another embodiment the invention relates to a vector comprising a DNA sequence according to the invention.

In a further embodiment, the invention relates to a vector comprising DNA sequences encoding at least two (poly)peptide/, comprising at least a hetero-associating (poly)peptide A_m and a hetero-associating (poly)peptide B_n.

Vectors which can be used in accordance with the present invention are well-known to the practitioner in the art.

In another embodiment, the invention relates to a host cell containing at least one vector of the present invention.

In a further preferred embodiment the host cell is a mammalian, preferably human cell, a yeast cell, an insect cell, a plant cell, or a bacterial, preferably E.coli cell.

In a highly preferred embodiment, the invention relates to a method for the production of a hetero-associating (poly)peptide, an optimized hetero-associating (poly), a (poly)peptide/protein, or a hetero-associated (poly)peptide/protein of the present

invention, which comprises culturing the host cell of the present invention in a suitable medium, and recovering said (poly)peptide or said (poly)peptide/protein produced by said host cell.

In a most preferred embodiment, the invention relates to a pharmaceutical composition comprising the hetero-associated (poly)peptide/protein or the present invention.

Still further preferred is a diagnostic composition comprising the hetero-associated (poly)peptide/protein of the present invention.

Further preferred is a kit containing at least one of
a hetero-associating (poly)peptide, an optimized hetero-associating (poly)peptide, or a (poly)peptide/protein of claims, or a hetero-associated (poly)peptide/protein of the present invention; or
a vector of claims according to the invention.

FIGURE CAPTIONS

Figure 1: (A) Schematic representation of a parallel dimeric coiled coil. Hatched bars indicate the possible interhelical interactions between e and g positions. (B) Schematic representation of the protein complementation assay as described in the Examples. Introduction of a mutation at the DHFR interface (I114A) was used to increase selection stringency (19). (C) Overview of the library design depicted as a helical wheel plot from the N- to the C-terminus (inside to outside. The randomized positions are indexed (*: equimolar mixture of Q, E, K, R, x: equimolar mixture of N, V). The selected residues from the predominant pair, WinZip-A1B1 (clone #1, Fig. 1D), are next to the randomized positions in the respective box. (D) Partial sequences (randomized positions) of clones from the highest stringency selection which survived at least until passage 10 (clone #1: WinZip-A1B1, clones #2 to #10: WinZip-A3B3 to WinZip-A11B11). Clone #1 (named WinZip-A1B1) was identified 18/22 times in passage 12 and 4/11 times in passage 10 (the full amino acid sequences are shown in Fig. 11).

Figure 2: (A) DNA constructs code for fusions between library proteins (shown as α-helical leucine zippers) and either fragment of murine DHFR (mDHFR). Fusions were created using either the wild-type or the mutant mDHFR fragment 2 (Ile114Ala), yielding LibA-DHFR[1] and LibB-DHFR[2] or LibB-DHFR[2:I114A], respectively. (B) Principle of the mDHFR-fragment complementation assay: *E. coli* cells are cotransformed with both fusion libraries in minimal medium, in the presence of IPTG (for induction of expression) and trimethoprim (for inhibition of the bacterial DHFR). If the library proteins heterodimerize, mDHFR can fold from the individual fragments resulting in active enzyme and bacterial growth. Both mDHFR fragments must be present, and dimerization of the fused proteins is essential, in order for cell propagation to be possible. No growth is observed if any of these conditions is not fulfilled (19) . The surviving colonies are the result of "single-step selection" and can be directly analyzed by DNA sequencing. (C) "Competition selection" is undertaken by pooling colonies from (B) in selective, liquid culture (passage 0 or P0), propagating the cells and diluting into

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fresh selective medium for further passages. An aliquot can be plated and the resulting colonies analyzed by DNA sequencing.

Figure 3: Competition selection and chain shuffling. (A) Approximately 1.42×10^4 clones resulting from single-step, I114A-mutant selection were pooled (=P0) and competition selection was undertaken as described in Figure 2C, and in the Examples. At each passage, some cells were plated and colony sizes were quantitated. (B) Quantitation of the colony sizes from (A). For comparative purposes, quantitation of colony sizes of cells transformed with DNA of WinZip-A1B1 (but not passaged in liquid culture) is shown. (C) Quantitation of the colony sizes from passages of the chain shuffling experiment: WinZip-B1-DHFR[2:I114A] + LibA-DHFR[1]. In (B) and (C) the numbers of colonies were normalized such that passages could be directly compared.

Figure 4: (A) Schematic representation of a leucine zipper pair visualized from the N-terminus illustrating e/g-interactions and the hydrophobic core formed by the a- and d-positions. (B) Distribution of residues at the semi-randomized positions throughout selection. The number of zipper pairs sequenced is given in parentheses, save "Before selection" where the theoretical distribution is reported. Each pair carries one core a-pair and 6 e/g-pairs. Neutral e/g-pairs have one or both residues as Gln. In "Competition (I114A)" only clones from P6 to P12 (not from earlier passages) were considered for analysis. Thus, 37 individual clones were sequenced, and most of the resulting sequences were identical in two or more clones. The distributions were calculated according to the frequency of sequence occurence (n=37). (C) Leucine zipper sequences obtained after competition selection and chain shuffling. The heptad positions (a to g) are followed by the heptad number (1 to 5). Invariant residues from GCN4 are underlined. Clear boxes indicate the semi-randomized e- and g-positions and core a-position (a3). Circled residues were designed to contribute to helix capping. Shaded residues were designed for the introduction of restriction sites. Other residues are from c-Jun (LibA) or c-Fos (LibB). Arrows indicate putative e/g-interactions.

Figure 5: Efficiency of competition in a model selection. The selection was set up by mixing known numbers of cells expressing either GCN4-DHFR[1]/GCN4-DHFR[2:I114A] fusions or one of 7 LibA-DHFR[1]/LibB-DHFR[2:I114A] pairs previously selected by single-step selection. The starting ratio was 2.9×10^4: 1(GCN4 to Lib). Competition selection was undertaken as described in Figure 2C, and in the Examples. The appearance of the library pairs in the pool was monitored by restriction analysis. A *Pvu*II fragment (1138 bp) is unique to the LibB sequence of the LibB-DHFR[2] plasmid, while another (762 bp) is from pRep4 (repressor plasmid) and remains approximately constant. The bands were quantitated using the NIH Image gel analysis function to calculate the ratio of LibB/pRep4 (indicated below each lane).

Figure 6: (A) Deviation of observed e/g-interactions in the selected heterodimer and the two putative homodimers from the statistically expected distribution in the absence of selection. Interactions are grouped in potentially attractive (E-K, K-E, E-R, R-E; left black bars), neutral (Q Q, Q-E, E-Q, Q-K, K-Q, Q-R, R-Q; grey bars) and repulsive (E-E, K-K, K R, R-K, R-R; right black bars). (i) Low stringency selection: clones were subdivided into those with an N-N pair in the core a-position (n=8) and those with an N-V or V-V pair (n=6), (ii) medium stringency selection: only clones with an Asn-pair in the core a-position were considered (n=23), and (iii) highest stringency selection: clones, which survived the competition selection at least up to passage 10 (Fig. 1D) were considered. These were analyzed counting each sequence once (not weighted, n=10) or according to their frequence of appearance (weighted, n=37). (B) Number of selected pairs having a certain difference of attractive (grey bars) or repulsive interactions (black bars), respectively, between the heterodimer and its constituting homodimers. The definition of (i), (ii) and (iii) is as in (A).

Figure 7: Positional distribution of amino acids at each e- and g-position in sequences obtained from the highest stringency selection. The statistically expected random occurrence of each amino acid at each position was subtracted from the relative occurrence observed in the selection (Q left (black), E middle (grey), K/R right (black)).

Figure 8: (A) Determination of the molecular weight of WinZip-A1B1 by sedimentation equilibrium. The upper panel shows the residuals between measured data obtained at 10 μM peptide concentration at 25°C and data fitted as monomer (top), dimer (middle), or trimer (bottom). The lower panel shows the residuals for a dimeric fit to the data set obtained at 150 μM peptide concentration, 10°C. (B) Native gel electrophoresis. 1: WinZip-A1 (pI 11.2); 2: WinZip-B1 (pI 4.9); 3: WinZip-A1B1. (C) Urea titration of the heterodimer WinZip-A1B1 (■), and the homodimers WinZip-A1 (▲) and WinZip-B1 (▼).

Figure 9: CD-measurements of the synthesized peptides of WinZip-A1B1. (A) Temperature dependence of $[\Xi]_{222}$ for WinZip-A1B1 (■), WinZip-A1 (▲), WinZip-B1 (▼), and the calculated average of both homodimers (– – –). (B) Dependence of T_m and ΔT_m (–◇ –) on pH, and (C) on salt. It must be noted that thermal denaturation of WinZip-A1 was not completely reversible at 1 M salt concentration, and only 71% of the starting signal was regained.

Figure 10: Sequencing profile of pools from passages of the chain shuffling WinZip-B1-DHFR[2:I114A] + LibA-DHFR[1]. Representative semi-randomized positions (see Fig. 4) were taken from a single competition experiment, such that the selection rates can be directly compared. The ratio of the individual triplet codons (central three nucleotides of each frame) was visually estimated (CAG = Gln; GAG = Glu; AAG = Lys; CGT = Arg; the equimolar random mix of the 4 codons results in the predominance of C at the first position, A at the second and G at the third). Mixed positions are marked by (NNN), positions where a single codon is dominant (> 50%) are marked in lower case and those where the codon is clear (> 90%) are marked in upper case. For passages 0, 2 and 8, two independent sequencing reactions were performed, which yielded identical results.

Figure 11: (A) Sequences of (poly)peptides WinZipA1 to WinZipA11

(B) Sequences of (poly)peptides WinZipB1 to WinZipB11

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The examples illustrate the invention:

EXAMPLES

All reagents used were of the highest available purity. Sequencing was carried out either by cycle sequencing with fluorescence labeling (MWG-Biotech, Ebersberg, Germany) using a LiCor detection system or by automated sequencing with an ABI sequencer. Restriction endonucleases and DNA modifying enzymes were from Pharmacia and New England Biolabs. *E. coli* strain XL1-Blue (Stratagene) was used for subcloning and propagation of the libraries. *E. coli* strain BL21 harboring the *lacI^q* plasmid pRep4 (Qiagen) was cotransformed with the appropriate DNA constructs for the survival assays.

Abbreviations: CD, circular dichroism; mDHFR, murine dihydrofolate reductase; WinZip: dominant zipper pairs obtained from competition selection; WinZip-A1B1: original pair selected, comprising peptide A1 from libraryA and peptide B1 from libraryB; WinZip-A1B2 and WinZip-A2B1: optimized pairs comprising the original partner A1 or B1 and the new partner B2 or A2, respectively.

Example 1: Selection of hetero-association peptides (see WO 98/34120, Example 7)

1.1   Introduction

Here we present a strategy for library-*vs*-library screening in intact cells based on the folding of murine enzyme dihydrofolate reductase (mDHFR) from complementary fragments (18, 19). DHFR was genetically dissected into two rationally designed fragments, each of which can be fused to a library of proteins or peptides (Fig. 2A).

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Members of one library which heterodimerize with a member of the other library drive the reassembly of the mDHFR fragments, resulting in reconstitution of enzymatic activity (Fig. 2B). Activity is detected *in vivo* using an *E. coli*-based selection assay, where the bacterial DHFR is specifically inhibited with trimethoprim, preventing biosynthesis of purines, thymidylate, methionine and pantothenate, and therefore cell division. The reconstituted mDHFR, which is insensitive to the low trimethoprim concentration present in selection, restores the biosynthetic reactions required for bacterial propagation. As a result, the interaction between library partners is directly linked to cell survival and detected by colony formation. We have previously demonstrated the utility of this strategy with GCN4 leucine zipper-forming peptides, as well as with larger heterodimerizing partner proteins (19) with K_Ds ranging between 3 and 160 nM (20, 21), although the affinity limits have not been determined.

1.2   Constructs for DHFR fragment complementation

The DNA constructs encoding the *N*-terminal (1-107) and *C*-terminal (108-186) mDHFR fragments have been previously described (19). The vectors are variants of plasmids Z-F[1,2], encoding the N-terminal DHFR fragment, and Z-F[3] or Z-F[3:I114A], respectively, encoding the C terminal DHFR fragment with or without the I114A mutation (19). Briefly, each fragment was amplified by PCR with appropriate unique flanking restriction sites and subcloned into a bacterial expression vector (pQE-32 from Qiagen). Each plasmid encodes an N-terminal hexahistidine tag, followed by a designed flexible linker and the appropriate DHFR fragment. Unique restriction sites between the hexahistidine tag and the flexible linker allow subcloning of the desired library.

1.3   Library design

Our goal was to select for metabolically stable dimeric coiled-coils with high heterospecificity. Thus, two libraries were designed to meet the requirements of genetic diversity to prevent recombination, high helix stability and a high probability of complementarity, all within a reasonable library size (Fig. 1C).

As templates for the outer, solvent exposed residues (positions b, c, f) we chose the

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leucine-zipper regions of the proto-oncogenes c-Jun and c-Fos for libraryA and B, respectively, thus minimizing potential recombination despite the repetitive pattern of the heptads. Indeed, no recombination was found in any of the 80 clones sequenced. We chose a helix length of 4.5 heptads as good compromise between stability and size. For the hydrophobic core residues (positions a, d) we chose the residues of the parallel, homodimeric leucine zipper GCN4 (Val at a, Leu at d). A single a-position in the middle of each helix is often occupied by a polar residue, most often an Asn, which forms a hydrogen bond inside the hydrophobic core (22, 23). Replacement of this Asn pair by a non-polar one increases the stability significantly, but leads to helices packing in different registers and orientations, as well as forming higher order oligomers (24-26). Since we could not ascertain a priori whether higher specificity or stability would be more advantageous, we included both by allowing Asn and Val at the core a-position with equal probability. A difficult problem in library design is to encode only the desired amino acids with a predetermined ratio. We solved this problem by using defined trinucleotide mixtures in the oligonucleotides, where each trinucleotide codes for one specific amino acid (27).

The solvent-accessible residues at the e- and g-positions can form interhelical salt bridges or hydrogen bonds which can contribute to stability and heteromeric specificity (13, 14, 28, 29). Based on these results and on commonly occurring amino acids at these positions (30, 31), we simultaneously randomized all eight e- and g-positions with equimolar mixtures of Gln, Glu, Lys, Arg, also using trinucleotide codons in DNA synthesis. Including the Asn-Val combination at the core a-position, each library had a theoretical diversity of 1.3×10^5.

To increase the stability of the helices, we introduced helix capping residues in both libraries to saturate the missing hydrogen bonds at the helix ends with their side chains. Based on studies of helix-capping propensities in proteins (32, 33) and peptides (34, 35), we chose T-X-X-Q (Ncap-N1-N2-N3) for libraryA, and S-X-X-E for libraryB. The C-cap has only a minor effect on helix stability (35). As Gly has a high preference for the C-cap position (32, 33), we added a C terminal Gly. This may contribute to helix

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termination or extend the linker which connects the coiled coil to the DHFR fragments (Fig. 1B).

1.4   Library synthesis

Trinucleotide codons (27) were used to code for randomized positions, all other positions were made with mononucleotides.

LibraryA: TACTGTGGCGCAACTGNNNGAANNNGTGAAAACCCTTNNNGCTNNNXXX-TATGAACTTNNNTCTNNNGTGAGCGCTTGNNNGAGNNNGTTGCCCAGCTTGCTA (encoding VAQLXEXVKTLXAXZYELXSXVQRLXEXVAQL, wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V);

libraryB: CTCCGTTGACGAACTGNNNGCTNNNGTTGACCAGCTGNNNGACNNNXXX-TACGCTCTGNNNACCNNNGTTCGCAGCTGNNNAAANNNGTGGAAAAGCTGTGATA (encoding VDELXAXVDQLXDXZYALXTXVAQLXKXVEKL, wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V)

(NNN = equimolar mixture of the trinucleotides AAG, CAG, GAG, CGT; XXX = equimolar mixture of the trinucleotides AAT, GTT).

Generation of the second strand and introduction of SalI and NheI restriction sites were achieved by PCR using the primers prA-fwd: GGAGTACTGGCATGCAGTCGACTACT-GTGGCGCAACTG and prA-rev: GGACTAGTACCTTCGCTAGCAAGCTGGGCAAC or prB-fwd: GGAGTACTGGCATGCAGTCGACCTCCGTTGACGAACTG and prB-rev: GGACTAGTGCTAGCTTCTGACAGCTTTTCCAC, respectively. This resulted in a 142 bp double-stranded oligonucleotide for either library.

1.5   Expression plasmids

LibraryA and B were both digested with SalI and NheI, gel purified and ligated to the appropriate vector (Fig 2) yielding the plasmids LibA-DHFR[1], LibB-DHFR[2], LibB-DHFR[2:I114A] (Fig. 2A). After subcloning, the resulting linker between either library and DHFR fragment was: A(SGTS)$_2$ STSSGI for LibA and SEA(SGTS)$_2$STS for LibB. To achieve maximal library representation, the ligation mixes were individually electroporated into XL1-Blue cells and selected with ampicillin on rich medium (LB). A 2-

to 7-fold over-representation of each library was obtained. The resulting colonies were pooled and the plasmid DNA purified such that supercoiled plasmid DNA was obtained for cotransformation. The supercoiled DNA was cotransformed in BL21 cells yielding about 4×10^6 double-transformants. We used BL21 cells with a transformation efficiency of no less than 5×10^7 transformants per mg of DNA using 200 pg of DNA, or 2×10^7 transformants per mg using 500 ng of DNA. In cotransformations, the occurrence of double transformation was calculated as the number of colonies growing under selective pressure with trimethoprim (described below) divided by the number growing in the absence, when cotransformed with equal amounts of each DNA of a given, pre-selected pair. In order to verify that the library populations encode the designed amino acids with the expected frequency, single clones from each library were randomly picked and sequenced before selection. No statistically significant biases were detected. Seventy to 80% of each library had no mutations or frame-shifts, and thus the library-vs-library combination yielded approximately 50% correct sequence combinations. Thus, the experimental library-vs-library size of correct pairs is estimated as 2×10^6.

1.6   Selection procedure.

Three selection strategies were tested here, each having a different level of stringency. In the lowest stringency selection, we screened two expressed libraries against each other in a *single-step selection* (Fig. 2B), where cells cotransformed with complementary libraries were directly plated on selective medium plates (M9 medium with 1 μg/ml trimethoprim), and resulting colonies were analyzed, thereby identifying all interacting polypeptide partners. In the second strategy, we increased the selection stringency by using a mutant DHFR fragment (Ile114Ala) containing the destabilizing I114A mutation in DHFR[2] which occurs at the interface between both DHFR fragments. This mutation prevents stable reassembly of DHFR from its fragments (19) and should thus require more efficiently heterodimerizing, as opposed to homodimerizing, interacting partners to drive enzyme reconstitution. Finally, we introduced competitive metabolic selection, where clones obtained with the second strategy were pooled and passaged through several rounds of *competition selection*, in order to enrich for the optimally

heterodimerizing partners. Thereby, the most stable heterodimers should have higher mDHFR activity and thus a growth advantage.

Selective pressure for DHFR was maintained throughout all steps by inhibiting the bacterial DHFR with trimethoprim (1 mg/ml) in minimal medium. Ampicillin and kanamycin (100 mg/ml and 50 mg/ml, respectively) were also included in all steps to retain the library plasmids and the $lacI^q$ repressor-encoding plasmid (pRep4), respectively. Expression of the proteins was induced with 1 mM IPTG. When selecting on solid medium, growth was allowed for 45 hrs at 37°C.

When it was necessary to control precisely the starting number of cells in a competition, the number of viable cells in the starter cultures was quantitated as follows. The appropriate clones were propagated in liquid media under selective conditions and dilute aliquots were frozen at -80°C with 15% glycerol. One aliquot for each clone was thawed and plated under selective conditions, and the colonies counted after 45 hrs. The volume of cells to use for P0 was then calculated, such that each clone should be over-represented by a factor of at least 2000. Colony sizes (in Fig. 3) were evaluated using the NIH Image Particle Analysis Facility. When selecting in liquid medium, the starting O.D. (600 nm) was either 0.0005 or 0.0001. Cells were propagated either in Erlenmeyer flasks or in a 10 liter New Brunswick fermentor, depending on the volume required to ensure adequate representation of all clones present, at 37°C with shaking, or stirring at 250 RPM. After 10 to 24 hrs, O.D. (600 nm) reached 0.2 to 1.0 and cells were harvested.

1.7   Single-step selection

As a first step in selection of heterodimerizing leucine zippers, a single-step selection was undertaken, using the wild-type mDHFR fragments, by cotransforming the libraries LibA-DHFR[1] and LibB-DHFR[2] and plating on selective media (Fig. 2B). This strategy applies only a low stringency of selection to the potential pairs, thus many library combinations were expected to be selected. Approximately 1.7% of the resulting ampicillin-resistant cells were doubly transformed, harboring (at least) one plasmid from each library when using 5 ng of each DNA, or 8% were doubly transformed when using

20 ng of each DNA, as seen from control transformations (calculated as described above). Of the doubly transformed cells which harbor no mutations or frame-shifts, approximately 35% formed colonies under selective conditions (Table 1). This result immediately demonstrates that even with relatively low stringency of selection, only a fraction of the possible combinations of the two libraries allows zipper heterodimerization leading to efficient mDHFR reassembly. Fourteen colonies resulting from two independent cotransformations were picked and the sequences encoding the zippers were determined. Even under these low stringency conditions there exist important sequence biases in these sequences relative to the unselected ones (Fig. 4B). A reduction in same-charged e/g-pairs from 31.3% (unselected) to 19% (selected) and an increase in opposite-charged pairs from 25% (unselected) to 31% (selected) were seen. As well, a strong enrichment of N-N pairing at the core a-position (25% unselected vs 57% selected) was observed. The characteristics that have been enriched are consistent with the selection of stable leucine zipper heterodimers.

1.8   Increased stringency: use of the mDHFR Ile114Ala mutation.

We repeated the single-step selection, using the Ile114Ala mutant of mDHFR (18, 19), in order to increase the stringency of selection. We reasoned that only library partners that form the most stable heterodimers can compensate for the reduced ability of the mDHFR(Ile114Ala) fragments to fold into active enzyme, resulting in higher enzyme activity and growth rates. When bacteria were cotransformed with LibA-DHFR[1] and LibB-DHFR[2:I114A], we observed a 50-fold decrease in the number of colonies upon selective plating compared to the wild-type DHFR fragments (Table 1). Twenty-five colonies were picked from 3 independent cotransformations and the DNA sequences were analyzed. The increase in selectivity was concomitant with an extremely strong selection for N-N pairing at the core a-position (92%; Fig. 4B), illustrating that the specificity of in-register parallel alignment provided by N-N pairing is more highly favored under these in-vivo selection conditions than the higher stability afforded by V-V pairing. Reassembly of mDHFR from its fragments requires that in the final structure, the two fragment N-termini be brought close enough together to allow native-like refolding of

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DHFR (see Fig. 1) (19, 36). The peptide linkers that connect the library sequences to the DHFR fragments must be sufficiently flexible to allow DHFR to fold from its fragments, but not so long that any C-terminal to N-terminal orientation of the final folded leucine zipper would be allowed. As a result of this structural requirement, parallel in-register heterodimerization of the library peptides is the only configuration possible. Other biases in these sequences were also more pronounced than with the wt DHFR fragments (Fig. 4B). In particular, an additional increase in opposite-charged e/g-pairs from 31% to 37% was seen. In one case, a point-mutation resulted in a single clone (1/25) with a V-T pair at the core a-position.

1.9   Competition selection: Efficiency of selection

To further increase the selection pressure, we applied the principle of competition selection. We reasoned that, among selected zipper pairs, those which result in more stable heterodimerization will allow the most efficient enzyme reconstitution, leading to higher DHFR activity. If DHFR activity is limiting for growth, the higher activity should result in more rapid bacterial propagation, hence these cells would become enriched in a pool. Thereby, after sequential rounds of growth-competition, subtle differences in growth rate can be amplified, increasing the stringency of selection relative to the single-step selection.

To determine the rate at which competition can enrich for particular partner pairs, we first set up a model competition with a limited number of clones as described in Figure 1C. The initial cell mixture (P0) contained known amounts of viable cells expressing either GCN4- DHFR[1]/GCN4-DHFR[2:I114A] or one of seven LibA-DHFR[1]/LibB-DHFR[2:I114A] pairs previously obtained in a single-step selection of those libraries, mixed at a ratio of $2.9 \times 10^4 : 1$ (GCN4 : library clones). Productive association of the homodimeric GCN4 pair should occur only 50% of the time versus up to 100% for heterodimerizing library clones, thus is disadvantaged. Within 3 passages, the library pairs were already visibly enriched (Fig. 5), and after 5 passages the measured ratio between a restriction fragment indicative of the library and a constant fragment from the repressor plasmid had reached its maximium, showing that enrichment was maximal.

Colonies resulting from passage 9 (P9) were sequenced. No GCN4 leucine zippers were present among 24 sequences analyzed. Therefore, enrichment of the library pairs over GCN4 by a factor of at least $24 \times 2.9 \times 10^4 = 7 \times 10^5$ was achieved. Four out of the 7 library clones initially present survived until P9, with varying distributions (data no shown). The experiment was also repeated at a lower starting ratio of GCN4 and the same library clones were enriched, consistent with their enrichement being truly the result of selection (and not of unrepresentative sampling). This indicated that selection among the pre-selected clones was not as rapid as that seen between pre-selected and GCN4 zippers, but that the smaller differences between the pre-selected ones can still be amplified in selection. These results demonstrate that there is a direct link between reconstitution of mDHFR and growth rate.

1.10 Competition selection for optimal pairs

Our ultimate goal was to select for the "best" among the zipper pairs obtained by single-step selection. We obtained a large initial number of clones by cotransforming bacteria with 0.5 mg of DNA each from LibA-DHFR[1] and LibB-DHFR[2:I114A]. Approximately 50% of cells were at least doubly transformed (52% + 10%, average of 2 independent control experiments, calculated as described in the Experimental Protocol). We obtained approximately 1.42×10^4 clones on selective medium, which arise from a 1.4×10^2-fold selection factor (see Table 1), and were thus selected from $(1.42 \times 10^4) \times (1.4 \times 10^2) = 2.0 \times 10^6$ library-vs-library cotransformants. These were pooled and passaged. There was a clear increase in colony sizes with subsequent passages, indicating that faster-growing clones were taking over (Fig. 3A, B). At P12, the colonies are homogeneously large, showing similar growth rates among the clones. Twenty-two individual colonies from P12 were picked and sequenced, as well as 11 from P10 and 2 from each previous second passage. A single pair (WinZip-A1B1, composed of WinZip-A1-DHFR[1] and WinZip-B1-DHFR[2:I114A]) was identified 18/22 times (82%) in P12, 4/11 (33%) in P10, but not in previous passages (Fig. 4C). While other sequences were found in early and late passages, none was as enriched as WinZip-A1B1. In order to verify that the growth rate recorded after competition (P12) was independent of bacteria-specific factors

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resulting from passaging, we cotransformed DNA from a pure clone of WinZip-A1B1 into fresh bacteria. The colony size distribution is similar for P12 and for the transformants (Fig. 3B), illustrating that the growth rate is a direct product of mDHFR reconstitution directed by the WinZip-A1B1 pair. The sequence bias observed at the core-a position was yet stronger here: only N-N pairing was recorded at the core a-position. When the biases at the e/g-positions were calculated according to the occurrence of each sequence (n=37), there was no significant change in opposite charged pairing (37%), while a small increase in same-charged pairing was observed (from 23% to 26%) as a result of the two same-charged pairs which occur in the predominant WinZip-A1B1 (Fig. 4B, C). However, when each unique sequence was considered only once (n=10) a further increase of opposite-charged e/g-pairing was observed.

Example 2: Analysis of clones resulting from library screening

2.1   Introduction
Clones resulting from the three selection strategies with increasing stringencies were analyzed and compared: (i) lowest stringency: 14 clones analyzed, (ii) medium stringency: 25 clones analyzed (see Table 2), (iii) highest stringency: 41 clones analyzed from various passages. The last passage (P12) yielded a population dominated by a single pair of coiled-coil sequences, WinZip-A1B1, as described above (Fig. 1C, clone #1 in Fig. 1D), which was biophysically analyzed (see below). The sequences of clones surviving at least up to passage 10 are reported in Figure 1D. By comparing the selected clones from the three strategies, we analyzed the preferences for the core a-position, the distribution of e/g-pair combinations, and the presence of any bias for certain amino acids within the individual helices.

2.2   Selection in the core a-position
Sequencing of 16 clones prior to selection showed an equal distribution of Asn and Val at the core a position. After selection a strong bias was found toward Asn-pairs, which

became stronger with increasing selection stringency. No bias was seen between Val-Val or Val-Asn combinations. An Asn-pair was found in 57% (n=14) of the lowest stringency, in 92% (n=25) of the medium and in 100% (n=41) of the highest stringency selection. Thus, the specificity gained by this polar interaction clearly outweighs the more energetically stable Val-pairs. This seems to be a very important feature, since a strong selection occurred even at the lowest stringency.

2.3   Selected e/g-pairs in hetero- and putative homodimers

All selected clones must form a heterodimer to reconstitute DHFR activity, however, the heterospecificity, and thus the ratio of heterodimers (libraryA/libraryB) to homodimers (libraryA/libraryA and libraryB/libraryB) can vary, and a mixture of both may still generate sufficient amounts of active enzyme to allow cell propagation under low stringency conditions. We analyzed the average occurrence of all e/g-pair combinations in the heterodimers and the putative homodimers arising from the various selections in relation to the random statistical distribution (Fig. 6A). The selected heterodimers show on average more attractive and less repulsive interactions than expected in a random population, indicating selection for stability. This trend, although increasing with higher stringency, is already observed in the lowest stringency selection, indicating that a certain threshold of stability is needed to induce enzyme dimerization. Selection for heterospecificity is achieved by a higher stability of the heterodimer relatively to the homodimers and is only observed in the medium and highest stringency selections using the destabilizing I114A DHFR[2]-mutant (compare Fig. 6A, (i) vs (ii) and (iii)). Interestingly, this effect is more pronounced for libraryA homodimers than for libraryB homodimers, and the biophysical characterization of WinZip-A1B1 indicated that the libraryA homodimer is more stable (see below), and thus might have a stronger influence on titrating out fragments than the libraryB homodimer.

To determine the degree of heterospecificity achieved in the various selections, the relative stability of heterodimer versus homodimer was estimated for each single clone. We calculated the difference of attractive or repulsive interactions, respectively, between the heterodimer and the average of the two corresponding homodimers and displayed a

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histogram of pairs as a function of this difference (Fig. 6B). The results clearly show that heterospecificity is achieved not only by a decrease of repulsive interactions but also by an increase of attractive interactions in the heterodimer relative to the homodimers. However, the lowest and medium stringency selection yielded still a certain fraction of pairs with no difference or even a reversed ratio, whereas the highest stringency selection exclusively yielded pairs with distinct heterospecificity. In addition, in no case were more than three repulsions found in the heterodimers, although in a random combination 8% of all pairs should have 4 to 6 repulsions.

2.4   Positional distribution of the selected amino acids

Intrahelical electrostatic interactions can influence stability and may even promote selection of apparently repulsive e/g-pairs. Interactions with the helix macrodipole, for example, can modulate stability in coiled coils (37). Indeed, we observed a bias for negatively charged and neutral amino acids in the N-terminal part and positively charged amino acids in the C-terminal part (Fig. 7). This positional preference may at least partially compensate the loss of stability resulting from a repulsive e/g-interaction. In addition, interactions with adjacent residues on the outside of the helix (b- and c positions) may influence the contributions of charges at the e- and g positions (38). This may explain why at position e1 in libraryB a negatively charged amino acid is not favored, contrary to the expected counterbalancing of the helix dipole, since this position is adjacent to two aspartates in positions b1 and b2. On a more general note, the predominantly selected sequence with the residues from c-Jun at the outer positions (libraryA) bears remarkable similarity to the e- and g sequences in the naturally-occurring c-Jun.

2.5   Library complexity

Although the predominantly selected sequence pair WinZip-A1B1 showed all the desired properties in vivo as well as in vitro (see below), we were not able to cover all theoretical library vs-library combinations in our selection. Nonetheless, we covered all possible electrostatic interaction combinations (+/+, -/-, +/-, +/n, -/n, n/n; n=neutral) in all six

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interacting e/g-positions, when grouping the core a-position into favored (Asn-Asn) or disfavored (Asn-Val, Val-Val) combinations. This reduces the theoretical library size from 1.7×10^{10} to 9×10^4 possibilities, which was well covered by the experimental library of 2×10^6. It is therefore likely that WinZip-A1B1 contains the most important features for stability and heterospecificity. Furthermore, the random probability of finding pairs with no repulsive interactions was 1:40, and with solely attractive interactions was $1:1.6 \times 10^4$. Thus, our selection covered a representative sequence space and the same-charged interactions in WinZip-A1B1 are not a result of incomplete library sampling but must have more subtle reasons, including in-vivo factors, which we cannot fully address. Furthermore, in the medium as well as in the highest stringency selection 13 out of 38 pairs sequenced had no repulsive e/g pairs, but none competed successfully against WinZip-A1B1 in the selection.

Example 3: Biophysical characterization of WinZip-A1, WinZip-B1 and WinZip-A1B1

3.1 Secondary structure and oligomerization state of the predominant pair WinZip-A1B1

We investigated the stability and specificity of the predominantly selected peptides WinZip-A1 and WinZip-B1 alone and in an equimolar mixture (WinZip-A1B1). All experiments were performed with N-acetylated and C-amidated synthetic peptides.

3.1.1 Peptide synthesis and purification

The peptides WinZip-A1: Ac-STTVAQLEEKVKTLRAQNYELKSRVQRLREQVAQLAS-NH2 and WinZip-B1: Ac-STSVDELQAEVDQLQDENYALKTKVAQLRKKVEKLSE-NH2 were synthesized (Applied Biosystem 431A) and purified by reversed-phase HPLC. Electrospray mass spectrometry confirmed purity and identity of the peptides with a mass deviation of less than 1 Da. Peptide concentrations were determined by tyrosine absorbance in 6 M GdnHCl (39).

3.1.2 Circular dichroism measurements

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All peptides formed stable α-helical coiled coils as demonstrated by CD-spectra.

CD spectra were recorded at 5°C at a total peptide concentration of 150 μM (1 mm cuvette, Aviv 62DS spectrometer). The standard buffer was 10 mM K_2HPO_4/KH_2PO_4, pH 7.0, 100 mM KF; salt concentration and pH were varied as indicated in the respective experiments. Thermal denaturations were measured at 222 nm in steps of 2.5°C (2 min equilibration, 30 s averaging). Thermal transitions were >91% reversible except where indicated. Apparent T_m were determined by least-squares curve fitting of the denaturation curves (40), assuming a two-state model. ΔT_m was calculated as T_m(WinZip-A1B1)-½[T_m(WinZip-A1)+T_m(WinZip-B1)]. Urea denaturation equilibria were determined at 20°C by automated titration of native peptide with denatured peptide in 6 M urea (30 μM WinZip-A1 or WinZip A1B1, respectively, or 60 μM WinZip-B1) measuring the CD signal at 222 nm (300 s equilibration, 30 s averaging). K_D values were calculated by linear extrapolation to 0 M denaturant assuming a two-state model (K_D=[unfolded monomer]^2/[folded dimer]).

The helical content was in the range of 90% (WinZip B1) to 100% (WinZip-A1 and WinZip-A1B1). Peptide WinZip-A1 as well as the mixture WinZip-A1B1 (Fig. 4A) were dimeric at 10°C and 25°C over a concentration range from 10 μM to 150 μM as determined by equilibrium sedimentation. WinZip-B1 was partially unfolded as seen both by CD (Fig. 8C at 0 M urea) and equilibrium sedimentation.

3.1.3 Equilibrium sedimentation.

Equilibrium sedimentation experiments were performed using a Beckman XL-A Ultracentrifuge. Absorbance was monitored at 220 and 275 nm at peptide concentrations of 10, 50 and 150 μM in 10 mM K_2HPO_4/KH_2PO_4, pH 7.0, 100 mM KCl. Partial specific volumes and solvent densities were determined as described (41). The data sets were fitted to single molecular masses of monomer, dimer and trimer.

Equilibrium sedimentation indicated a mixture of monomers and dimers, with decreasing amount of dimer at increasing temperature.

3.1.4 Structural stability and heterospecificity

Thermal denaturation studies at neutral pH (Fig. 9A) revealed apparent T_m values of

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28°C (WinZip-B1), 49°C (WinZip-A1), and 55°C for the equimolar mixture of both (WinZip-A1B1). The large difference between the denaturation curve of the heterodimer and the average of the curves from WinZip-A1 and WinZip-B1 indicates that heterodimers form preferentially at equilibrium. This high heterospecificity is best reflected in a large and positive ΔTm value, and indeed, we observed a ΔT_m of 16.5°C.

To probe the mechanism of specificity, the effects of pH (Fig. 9B) and ionic strength (Fig. 9C) were investigated. All peptides were more stable at high pH, most likely because all have at least one e/g-pair with two positive charges which are neutralized at high pH. The increased stability of WinZip-B1 at low pH could be due to the shielding of electrostatic repulsions resulting from its high concentration of acidic residues. However, the ΔT_m is positive over the whole pH range indicating heterospecificity. The maximum degree of heterospecificity was observed at neutral to slightly basic pH, consistent with the intracellular pH of E. coli (42). High salt concentrations increased the absolute T_m values (Fig. 9C), presumably due to increased hydrophobic interaction or reduced electrostatic repulsion. However, the ΔT_m is reduced compared to low salt concentrations (0-100 mM), most likely due to the decreased influence of ionic interactions at higher ionic strength.

Interestingly, the overall stability did not correlate directly with the number of potentially repulsive e/g-interactions. The homodimer WinZip-B1 has two same-charged ion pairs, but is significantly less stable than the homodimer WinZip-A1 with four same-charged pairs (Fig. 9). Since the overall helical propensity is comparable for both peptides according to (43), the difference is probably due to intrahelical interactions. LibraryB might be destabilized by its high local concentration of acidic residues at the N-terminus. This may also explain why libraryA homodimers have generally more repulsive and less attractive e/g-interactions than libraryB homodimers, since the e/g-positions play a bigger role in the destabilization of the intrinsically more stable libraryA in order to reduce homodimerization.

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3.1.5 Native gel electrophoresis

Heterospecificity also was observed by native gel electrophoresis (Fig. 8B).

Gels (7.5% polyacrylamide (19:1), 375 mM ß-alanine acetate buffer, pH 4.5) were run with 500 mM ß-alanine acetate buffer, pH 4.5. Samples (~10 μg peptides per lane) were two-fold diluted with 600 mM ß-alanine acetate, pH 4.5, 0.2% methylgreen, 10% glycerol. Gels were prerun at 100 V for at least 45 min, run for 2-3 h at 5°C, and fixed with 2% glutaraldehyde or 20% (w/v) TCA, respectively, before staining with Coomassie blue.

To obtain a significant migration, an acidic buffer (pH 4.5) had to be used, and thus conditions where the heterospecificity is the lowest (ΔTm of only 7°C, compared to 16.5°C at neutral pH, Fig. 9C). Nevertheless, even under these stringent conditions heterodimers were obtained almost exclusively from the equimolar mixture (Fig. 8B), suggesting very high heterospecificity at neutral pH, and thus indicating how strongly heterospecificity was selected for.

3.1.6 K_D determination

Dissociation constants of the peptides were derived from equilibrium urea denaturations (Fig. 8C). The heterodimer WinZip A1B1 was the most stable species with a K_D of approximately 24 nM, while the homodimer WinZip-A1 had a K_D of approximately 63 nM. The accuracy of the K_D determination of WinZip-B1 is lower since it is already partially unfolded without denaturant (see above). The K_D was estimated to be in the 10^{-5} M range. Calculations were confirmed by determining the K_D values from thermal denaturation curves by a van't Hoff analysis, assuming as a first approximation a constant ΔH (40). We found reasonable agreement to the data obtained by urea denaturation with a maximal deviation of K_D by a factor of 2.6.

3.1.7 Comparison to other coiled coils

Designed coiled coils are usually only judged for being stable in vitro and, in certain cases, for heterospecificity, whereas naturally-occurring coiled coils must also function reliably in a cellular environment. Similar demands are imposed on our selection and on

further in-vivo applications, and therefore WinZip-A1B1 is best compared with other naturally-occurring coiled coils. The homodimeric coiled coil of the yeast transcription factor GCN4 has an equal or slightly higher Tm, depending on the length and concentration of the peptides chosen (44, 45). The N terminal homodimeric coiled coil of the APC protein has a Tm lower by at least 9°C than WinZip-A1B1 (46). The heterodimeric coiled coil from c-Jun/c-Fos shows comparable Tm and ΔT_m values (2). However, those data were derived from disulfide-bridged peptides. The coiled coil from c-Myc/Max also heterodimerizes to a fairly high extent, but peptides of comparable length have a T_m of only 31°C and a K_D of 60 μM (25°C) (45), whereas our WinZip-A1B1 has a Tm of 55°C and a K_D of approximately 24 nM (20°C). Thus, WinZip-A1B1 compares successfully with naturally-occurring coiled coils and will therefore be very useful for a variety of in-vivo applications.

Example 4: Chain shuffling of the WinZip-A1B1 sequences

3.1   Introduction

In the above experiment, WinZip-A1B1 was selected from a sample representing 2.0 x 10^6 library-vs-library cotransformants. As the theoretical library-vs-library diversity is $(1.31 \times 10^5)^2 = 1.72 \times 10^{10}$, approximately 0.01% of the library-vs-library space was sampled. However, we obtained a very high coverage of either single library (theoretical complexity of 1.31 x 10^5), where the probability of all members being present at least once is P=0.973. Thus, each polypeptide sampled only a small portion of the opposite library (2.0 x 10^6/ 1.31 x 10^5 = 15.4 polypeptides of the other library with P=0.999, assuming equal transformation rates for both libraries) and it is likely that better combinations for the WinZip-A1B1 peptides may be found. Using WinZip-A1B1 as a partially optimized starting point, we combined each of the two WinZip-A1B1 polypeptides with the opposite library (WinZip-A1-DHFR[1] + LibB-DHFR[2:I114A] and WinZip-B1-DHFR[2:I114A] + LibA-DHFR[1]).

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3.2   Chain shuffling

DNA from the WinZip-A1B1 clone was isolated and retransformed into bacteria in order to obtain clones carrying either plasmid WinZip-A1-DHFR[1] or WinZip-B1-DHFR[2:I114A]. A pure clone (for each) was electroporated with the appropriate library. Library representation was calculated by comparison with control transformations of the same cells with DNA from the other WinZip-A1B1 polypeptide (calculated as the number of colonies growing in the presence of trimethoprim divided by the number growing in the absence).

Single-step selection yielded pre-selected pools for either competition. In both cases, the library (1.3×10^5) was over-represented by a factor of 24 and 14, respectively, and the probability that all members were present at least once as partners of the "constant" peptide is P > 0.999 and 0.882, respectively. With passages of selection competition, a clear increase in colony sizes was again observed, indicating that faster-growing clones were taking over (Fig. 3C).

3.2   Analysis

At P0 and each second passage, DNA from the entire pool of cells was sequenced in order to follow the rate of evolution of each library against a constant partner. Figure 10 illustrates the results from representative semi-randomized positions. It is clear that the rate of selection is not constant at all positions: some positions showed a dominant residue (> 50%) already at P4 and clear selection (> 90%) at P6 (see position e2) while others remained mixed (<50%) until P6 and became clear only at P10 (see position g3). This was observed in both selections. The sequences from individual colonies were analyzed. In both selections, a predominant clone was identified (Table 1 and Fig. 4C), which is similar, but not identical, to the originally selected WinZip-A1B1 pair. The selection of the predominant clone WinZipA2B1 (selection of LibA-DHFR[1] against WinZip-B1- DHFR[2:I114A]) was achieved before P10, as P10 (4 clones analyzed) and P12 (4 clones analyzed) revealed only this clone. The selection of the predominant clone WinZipA1B2 (selection of LibB-DHFR[2:I114A] against WinZip-A1-DHFR[1]) was

clear but not complete after 12 passages, as it was identified 4/6 times in P12 and 3/5 times in P10.

Analysis of the biophysical properties of the peptide pairs selected in the chain shuffling experiments, performed as described in Examples 2 and 3, indicated that the observed e/g-interaction pattern is similar to that of WinZip-A1B1.

Conclusion

We applied a fast and simple strategy, a library-vs-library selection with the fragment complementation assay, to select for a metabolically stable, dimeric and highly heterospecific coiled coil with high affinity. Comparison of the outcome of various selections performed with different stringencies revealed insight into which properties are selected already at lower stringency, and are thus the most crucial for successful heterodimerization, and those which only become apparent at higher selection stringency, and thus represent a more subtle optimization. The most striking selection occurred at the core a-position for Asn-pairs, revealing that structural uniqueness is essential for efficient and selective heterodimerization. Furthermore, comparison of selected e/g-pairs from hetero- and putative homodimers indicated selection for stability even at the lowest stringency, whereas selection for heterospecificity was more pronounced at higher stringency. Heterospecificity was achieved not only by decreasing the numbers of repulsive e/g-interactions but also by increasing the number of attractive interactions in the heterodimer relative to the homodimers.

The selection for heterospecificity (and thus against homodimers) may be a unique feature of this selection system. Not only is active enzyme exclusively formed by parallel heterodimers, but homodimers and higher oligomers are likely to have a negative effect by unproductively wasting fragments and perhaps even harmfully accumulating non-functional enzyme. Dimer stability, in turn, is dependent not only on e/g-pair interactions, but also on helical propensity, intrahelical interactions and helix dipole stabilization. Indeed, our analysis revealed that the most successful variants do not simply consist of complementary charges in the e/g-positions, but show a more complicated pattern, presumably fulfilling a variety of naturally conflicting demands on the sequence, whose

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optimum would have been extremely challenging to predict.

The biophysical characterization of the predominantly selected pair WinZip-A1B1 revealed the formation of a stable dimeric coiled coil with very high heterospecificity, confirming the results from the sequence analysis and the validity of the selection strategy. The results obtained with WinZip-A1B1 and the peptide pairs identified in the chain shuffling experiment, are supporting the view that idealized sequences, based on the single principle of merely relieving repulsive e/g-interactions in the homodimers with complementary charges in the heterodimer, may not be optimal for biological applications

TABLE 1

TABLE 1: Stringency of the selection steps: selection factors.

Single-step selection	Selection factor[a]
Wt mDHFR fragments (5 or 20 ng)	2.8 ± 1.5
I114A mDHFR fragments (5 or 20 ng)	$(1.4 \pm 0.45) \times 10^2$

Competition selection	Initial Diversity	Frequency of dominant pair at P12[b]	Selection factor[c]
Competition (I114A)	3.9×10^6	WinZipA1-B1: 18/22 (82%)	3.2×10^6
Shuffling: WinZip-A1 + LibB-DHFR[2:I114A]	1.3×10^5	WinZipA1-B2: 4/6 (67%)	8.7×10^4
Shuffling: WinZip-B1 + LibA-DHFR[1]	1.3×10^5	WinZipA2-B1: 4/4 (100%)	$\geq 1.3 \times 10^5$

[a] The selection factor in single-step selection is defined as the number of cotransformed cells plated (considering only the 50% which give combinations with no mutations or frame-shifts), divided by the number of colonies surviving under selective conditions (see Results); average of 2 independent experiments, given with the standard deviation. This value must be calculated at low DNA concentrations (≤ 20 ng of each DNA) since the multiple cotransformations occuring at high DNA concentrations mask the actual selection factor. [b] P12 is the 12th round of serial cell passaging and competitive growth. [c] The selection factor in competition selection is defined as the proportion of the dominant pair multiplied by the sequence diversity it was selected from, and is the result of a single experiment.

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CLAIMS

1. A method for the identification of hetero-associating (poly)peptides comprising the steps of:

   (a)   providing a library A of (poly)peptides/proteins comprising (poly)peptides A_m having the general formula:

      VAQLXEXVKTLXAXZYELXSXVQRLXEXVAQL

   wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V

   (b)   providing a library B of (poly)peptides/proteins comprising (poly)peptides B_n having the general formula:

      VDELXAXVDQLXDXZYALXTXVAQLXKXVEKL

   wherein X represents a mixture of E, K, Q, and R, and wherein Z represents a mixture of N and V;

   (c)   combining in a common medium the (poly)peptides/proteins of said libraries A and B; and

   (d)   screening or selecting for a screenable or selectable property caused by the hetero-association of a (poly)peptide A_m with a (poly)peptide B_n.

2. The method of claim 1 wherein said libraries A and B are provided by providing libraries of nucleic acid sequences encoding said (poly)peptides/proteins, followed by causing or allowing the expression of said libraries of (poly)peptides/proteins.

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3. The method of claim 2 wherein said common medium are host cells, each cell harbouring nucleic acid sequences encoding a (poly)peptide/protein of each of said libraries A and B.

4. The method of claim 3 wherein said (poly)peptides/proteins of said libraries A and B further comprise either a N- or a C-terminal fragment of the murine DHFR enzyme, and wherein said screenable or selectable property is insensitivity of the host cell to trimethoprim by reconstitution of the DHFR enzyme on hetero-association of (poly)peptides A_m and B_n.

5. A hetero-associating (poly)peptide A_m taken from the list of:

    (a)   WinZipA1: VAQLEEKVKTLRAQNYELKSRVQRLREQVAQL

    (b)   WinZipA2: VAQLRERVKTLRAQNYELESEVQRLREQVAQL

    (c)   WinZipA3: VAQLQEKVKTLRARNYELKSEVQRLEEKVAQL

    (d)   WinZipA4: VAQLEEQVKTLQARNYELKSKVQRLKEKVAQL

    (e)   WinZipA5: VAQLEERVKTLRAQNYELKSKVQRLEEQVAQL

    (f)   WinZipA6: VAQLEEQVKTLEAENYELKSKVQRLRERVAQL

    (g)   WinZipA7: VAQLQEQVKTLEAQNYELESEVQRLKEQVAQL

    (h)   WinZipA8: VAQLEERVKTLKAENYELESEVQRLKERVAQL

    (i)   WinZipA9: VAQLEEKVKTLKAKNYELKSKVQRLKEKVAQL

    (j)   WinZipA10: VAQLQEEVKTLQAENYELRSEVQRLEEEVAQL

    (k)   WinZipA11: VAQLRERVKTLRARNYELQSKVQRLKERVAQL

6. A hetero-associating (poly)peptide B_n taken from the list of:

    (a)   WinZipB1: VDELQAEVDQLQDENYALKTKVAQLRKKVEKL

    (b)   WinZipB2: VDELKAEVDQLQDQNYALRTKVAQLRKEVEKL

    (c)   WinZipB3: VDELEAEVDQLKDQNYALKTKVAQLQKQVEKL

    (d)   WinZipB4: VDELRAKVDQLQDENYALETEVAQLQKRVEKL

    (e)   WinZipB5: VDELEAEVDQLEDQNYALQTRVAQLEKRVEKL

    (f)   WinZipB6: VDELKAKVDQLKDKNYALRTKVAQLRKKVEKL

(g)   WinZipB7: VDELRAQVDQLQDKNYALRTRVAQLKKRVEKL

(h)   WinZipB8: VDELQAEVDQLQDQNYALRTQVAQLKKKVEKL

(i)   WinZipB9: VDELRAQVDQLEDQNYALETQVAQLEKEVEKL

(j)   WinZipB10: VDELQAKVDQLKDENYALQTKVAQLQKRVEKL

(k)   WinZipB11: VDELRAEVDQLEDENYALRTRVAQLRKQVEKL

7.   A method for the identification of optimized hetero-associating (poly)peptides by using a hetero-associating (poly)peptide of claims 5 or 6 in a method according to anyone of claims 1 to 4, wherein a hetero-associating peptide WinZipA$_m$ of claim 5 is used instead of library A of (poly)peptides/proteins comprising (poly)peptides A$_m$ in step (a) of claim 1, or wherein a hetero-associating peptide WinZipB$_n$ of claim 6 is used instead of library B of (poly)peptides/proteins comprising (poly)peptides B$_n$ in step (b) of claim 1.

8.   An optimized hetero-associating (poly)peptide obtainable by the method of claim 7.

9.   A pair of hetero-associating (poly)peptides taken from the list of:

(a)   WinZipA1 and WinZipB1

(b)   WinZipA2 and WinZipB1

(c)   WinZipA1 and WinZip B2

(d)   WinZipA3 and WinZip B3

(e)   WinZipA4 and WinZip B4

(f)   WinZipA5 and WinZip B5

(g)   WinZipA6 and WinZip B6

(h)   WinZipA7 and WinZip B7

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(i)   WinZipA8 and WinZip B8

(j)   WinZipA9 and WinZip B9

(k)   WinZipA10 and WinZip B10

(l)   WinZipA11 and WinZip B11

10.   A (poly)peptide/protein comprising one of the hetero-associating (poly)peptides of claims 5 or 6, or an optimized hetero-associating (poly)peptide of claim 8, and a further (poly)peptide/protein.

11.   A (poly)peptide/protein of claim 10 wherein said further (poly)peptide/protein is an enzyme, a toxin, a cytokine, a metal binding domain, a transcription factor, a member of the immunoglobulin superfamily, a bioactive peptide of 5 to 15 amino acid residues, a peptide hormone, a growth factor, a lectin, a lipoprotein, a peptide which is able to bind to an independent binding entity, or a functional fragment of any said further (poly)peptide/protein.

12.   A hetero-associated (poly)peptide/protein comprising at least two (poly)peptide/proteins of claims 10 or 11, associated by hetero-association of a hetero-associating (poly)peptide A_m and a hetero-associating (poly)peptide B_n.

13.   A DNA sequence encoding a hetero-associating (poly)peptide of claims 5 or 6, an optimized hetero-associating (poly)peptide of claim 8, or a (poly)peptide/protein of claims 10 or 11.

14.   A DNA sequence encoding a hetero-associating (poly)peptide wherein said DNA sequence hybridizes under stringent conditions to a DNA sequence encoding a hetero-associating (poly)peptide of claims 5 or 6.

15. A vector comprising a DNA sequence of claim 13 of 14.

16. A vector comprising DNA sequences encoding at least two (poly)peptide/proteins of claims 10 or 11, comprising at least a hetero-associating (poly)peptide A_m and a hetero-associating (poly)peptide B_n.

17. A host cell containing at least one vector of claims 15 or 16.

18. A host cell of claim 17 which is a mammalian, preferably human cell, a yeast cell, an insect cell, a plant cell, or a bacterial, preferably E.coli cell.

19. A method for the production of a hetero-associating (poly)peptide of claims 5 or 6, an optimized hetero-associating (poly)peptide of claim 8, a (poly)peptide/protein of claims 9 or 10, or a hetero-associated (poly)peptide/protein of claim 12, which comprises culturing the host cell of claims 17 or 18 in a suitable medium, and recovering said (poly)peptide or said (poly)peptide/protein produced by said host cell.

20. A pharmaceutical composition comprising the hetero-associated (poly)peptide/protein of claim 12.

21. A diagnostic composition comprising the hetero-associated (poly)peptide/protein of claim 12.

22. A kit containing at least one of
   (a) a hetero-associating (poly)peptide of claims 5 or 6, an optimized hetero-associating (poly)peptide of claim 8, or a (poly)peptide/protein of claims 10 or 11, or a hetero-associated (poly)peptide/protein of claim 12; or
   (b) a vector of claims 16 or 17.

Figure 1:

A   Side view   Top view

B

C

D

clone (occurrence)	LibraryA									LibraryB								
	e1	g1	e2	g2	a3	e3	g3	e4	g4	e'1	g'1	e'2	g'2	a'3	e'3	g'3	e'4	g'4
#1 (22x)	E	K	R	Q	N	K	R	R	Q	Q	E	Q	E	N	K	K	R	K
#2 (3x)	Q	K	R	R	N	K	E	E	K	E	E	K	Q	N	K	K	Q	Q
#3 (2x)	E	Q	Q	R	N	K	K	K	K	R	K	Q	E	N	E	E	Q	R
#4 (1x)	E	R	R	Q	N	K	K	E	Q	E	E	E	Q	N	Q	R	E	R
#5 (1x)	E	Q	E	E	N	K	K	R	R	K	K	K	K	N	R	K	R	K
#6 (3x)	Q	Q	E	Q	N	E	E	K	Q	R	Q	Q	K	N	R	R	K	R
#7 (2x)	E	R	K	E	N	E	E	K	R	Q	E	Q	Q	N	R	Q	K	K
#8 (1x)	E	K	K	K	N	K	K	K	K	R	Q	E	Q	N	E	Q	E	E
#9 (1x)	Q	E	Q	E	N	R	E	E	E	Q	K	K	E	N	Q	K	Q	R
#10 (1x)	R	R	R	R	N	Q	K	K	R	R	E	E	E	N	R	R	R	Q
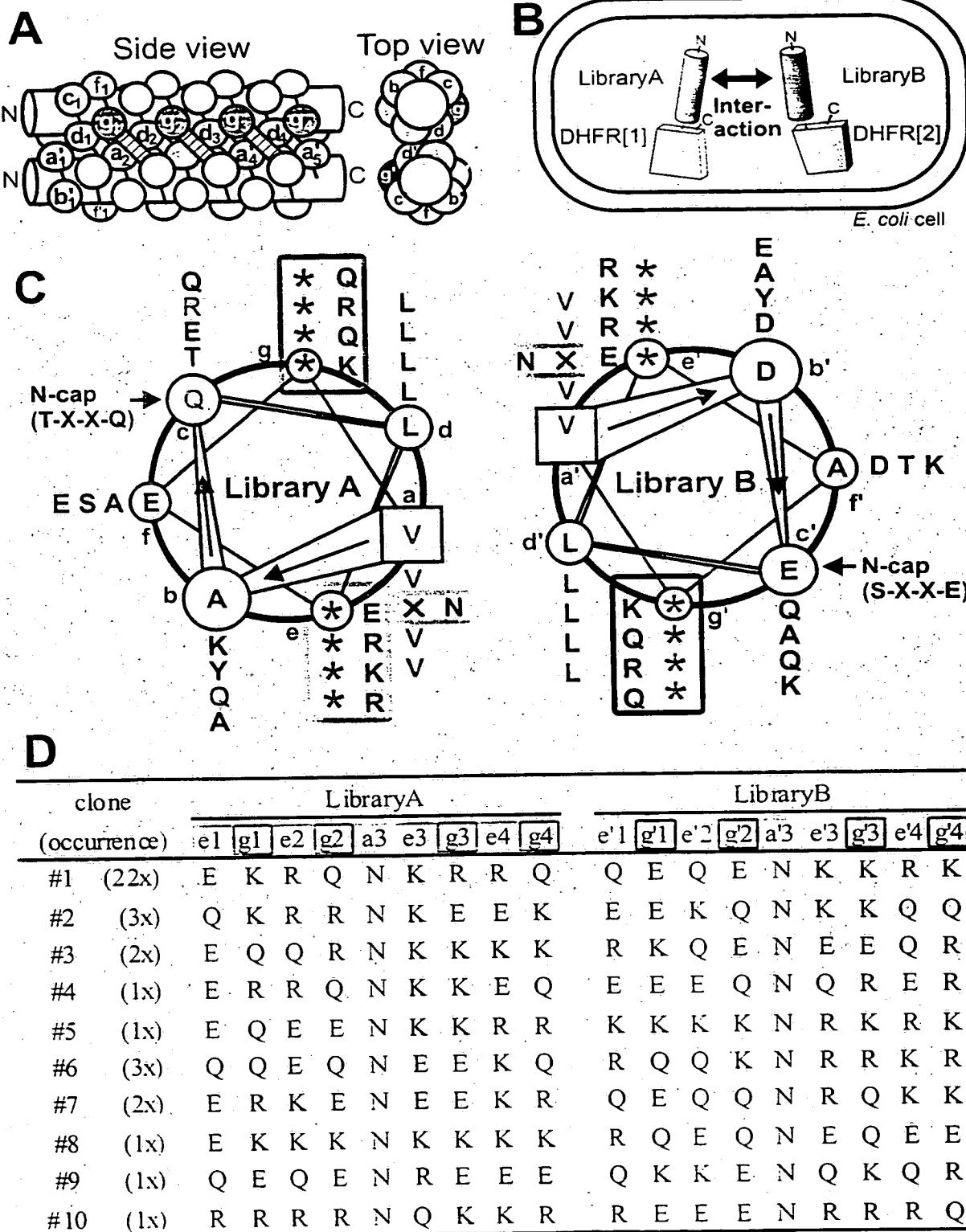
Figure 2:

(A) Library constructs
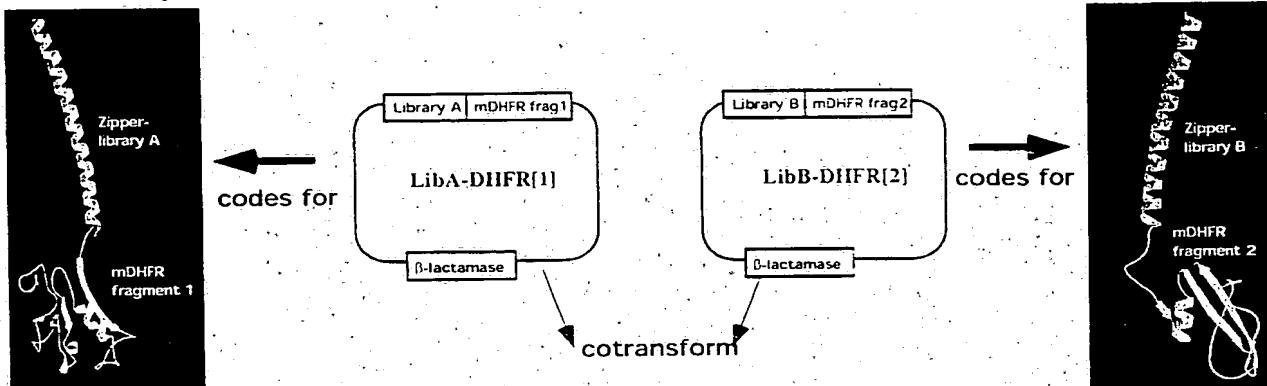
(B) Single-step selection
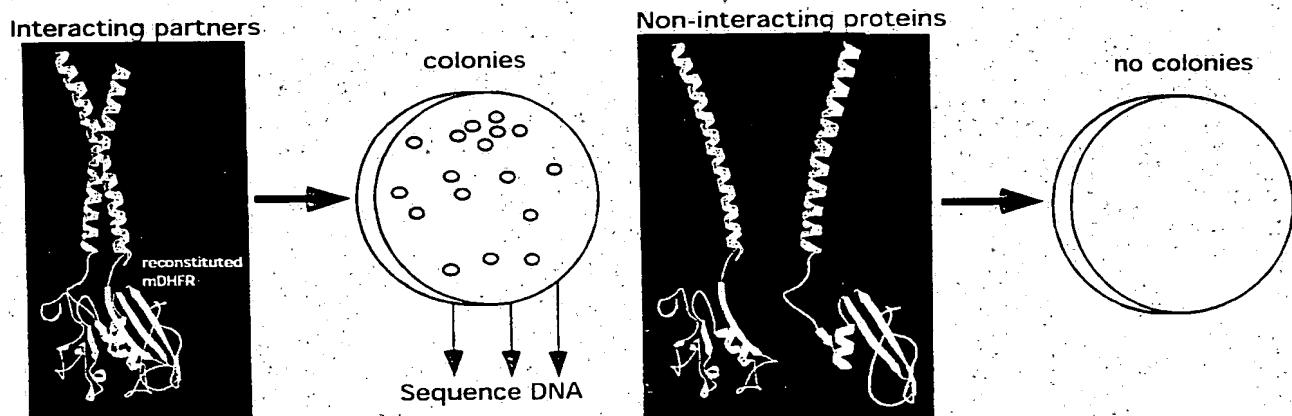

**(C) Competition selection**
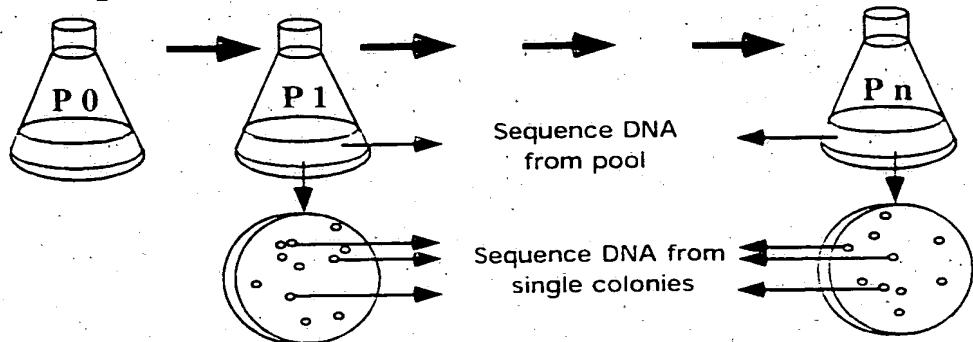
Figure 3:

Figure 4:

Figure 5:

Lib (1138 bp)—

pRep4 (762 bp)—

Ratio Lib/pRep4 (0) < 0.05 0.47 0.73 2.0 2.1 2.2 2.2 2.3

Figure 6:

difference in number of attractive (grey) or repulsive (black) interactions between heterodimer and homodimers
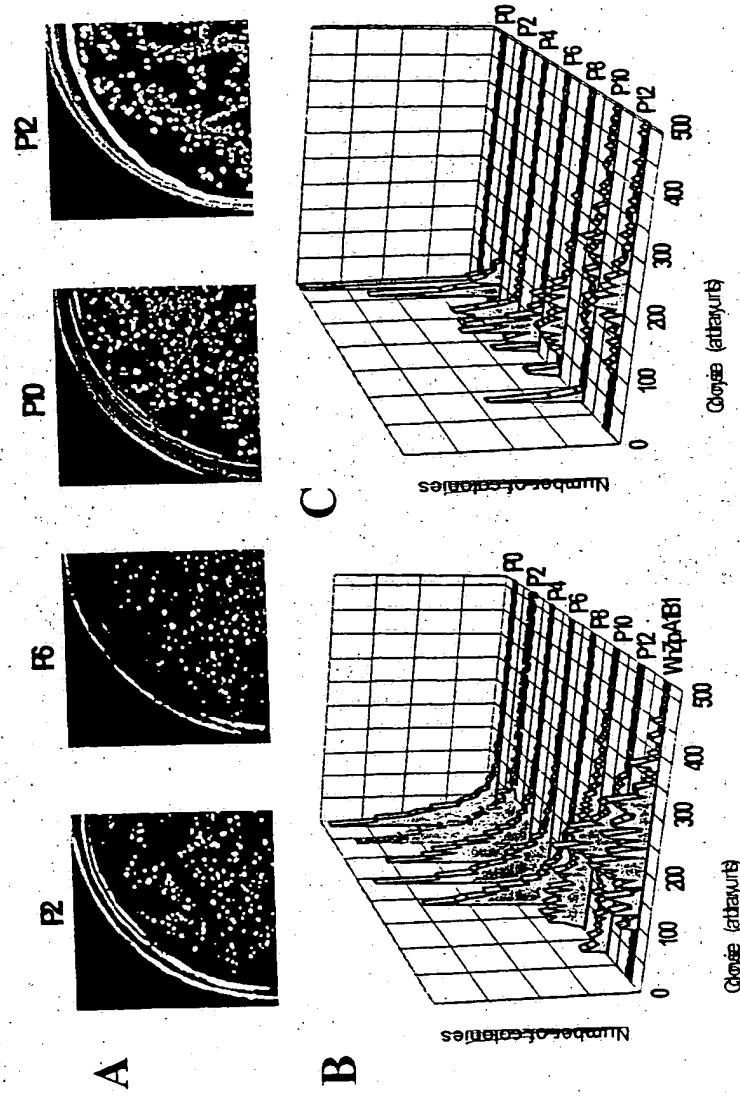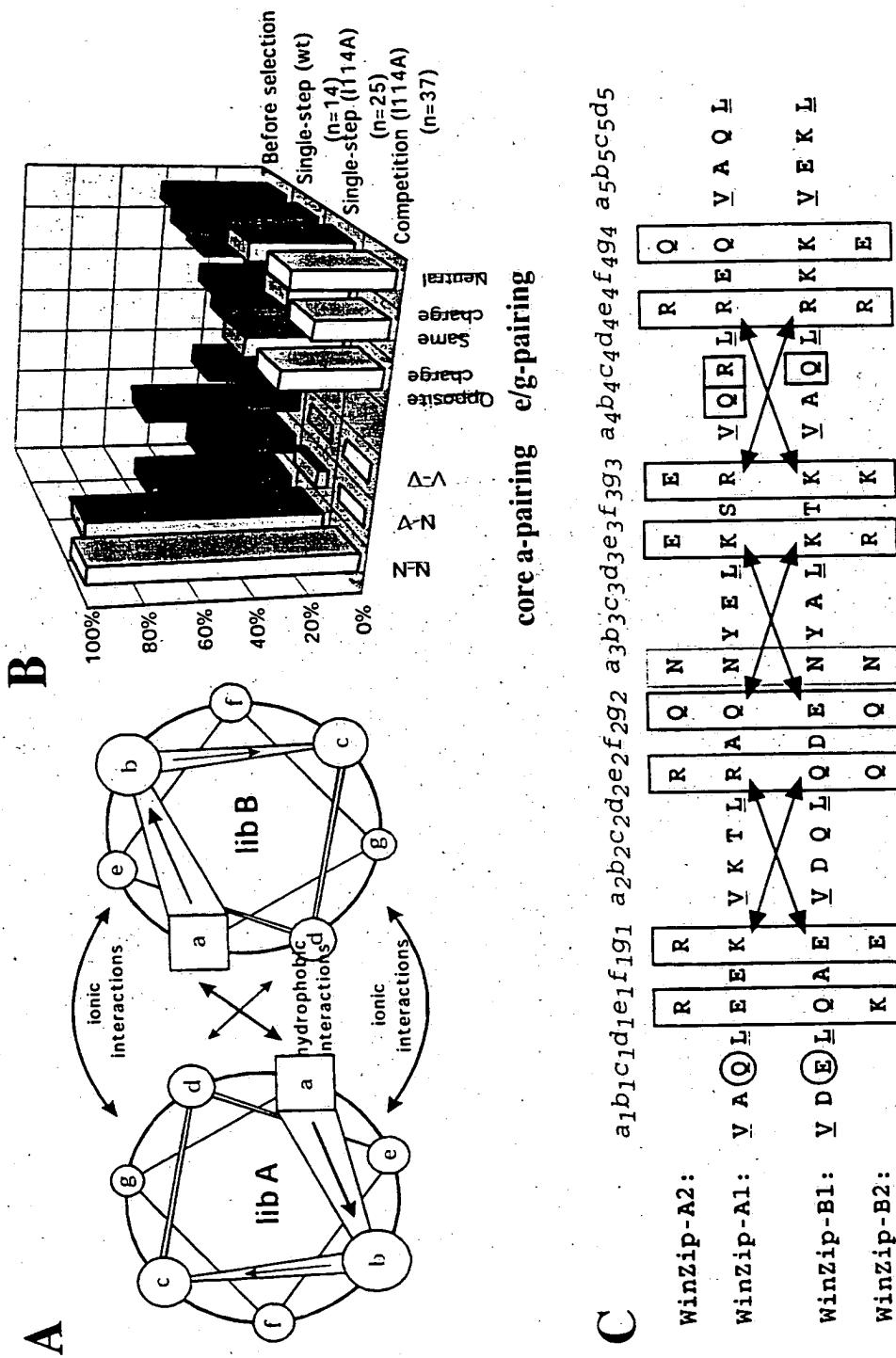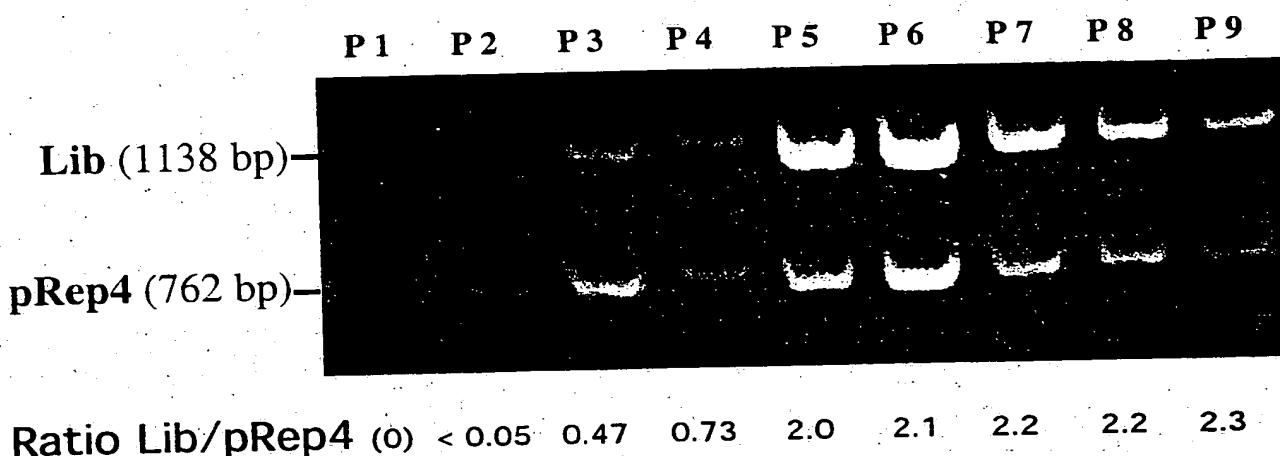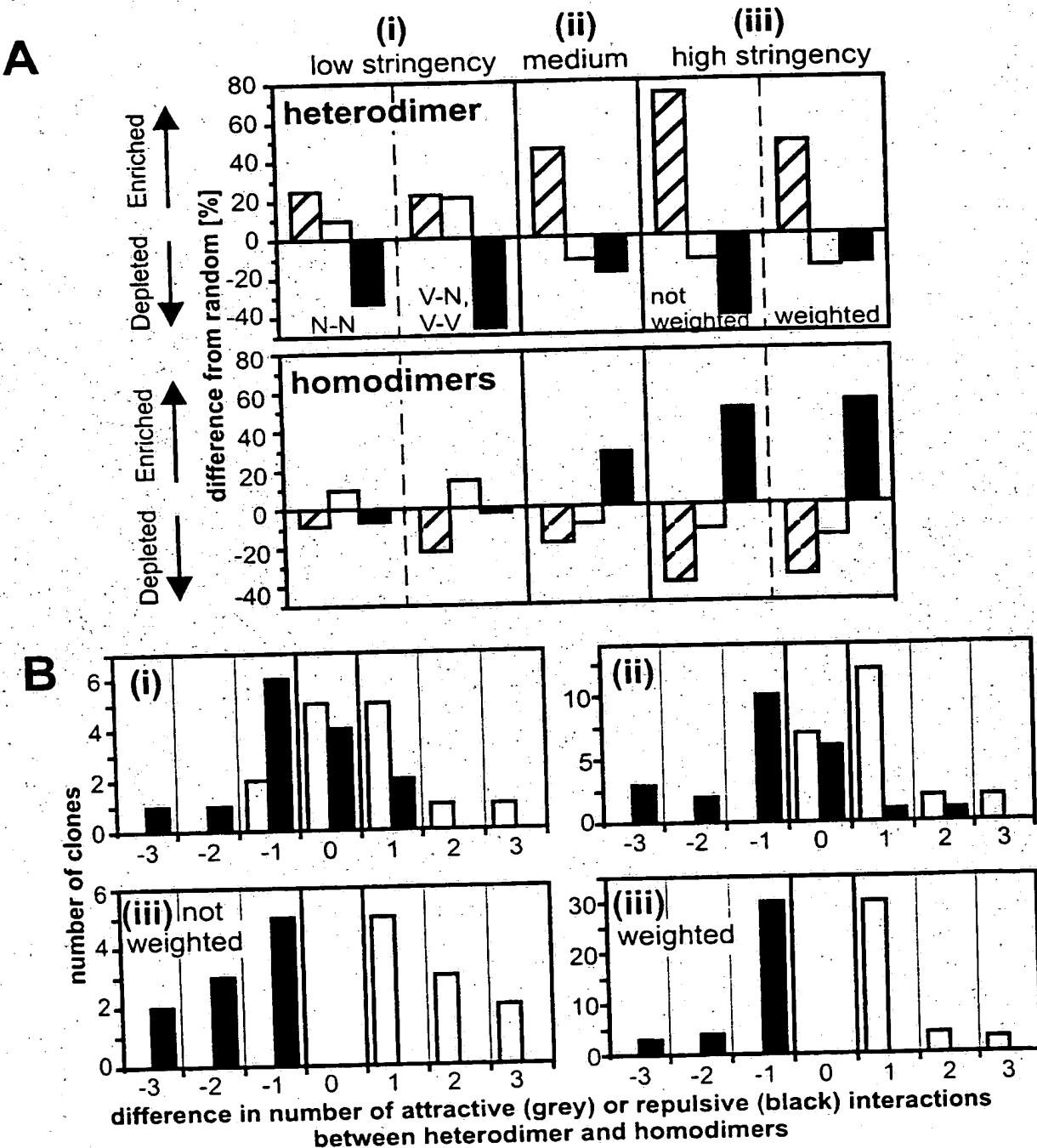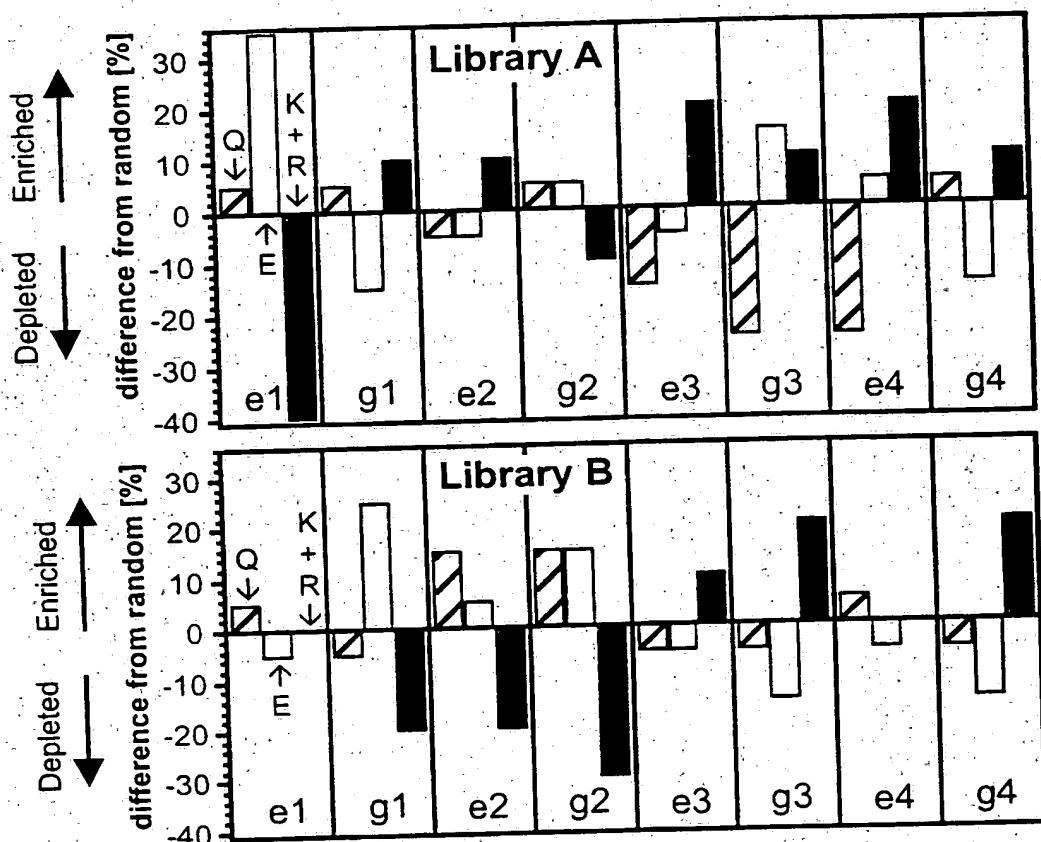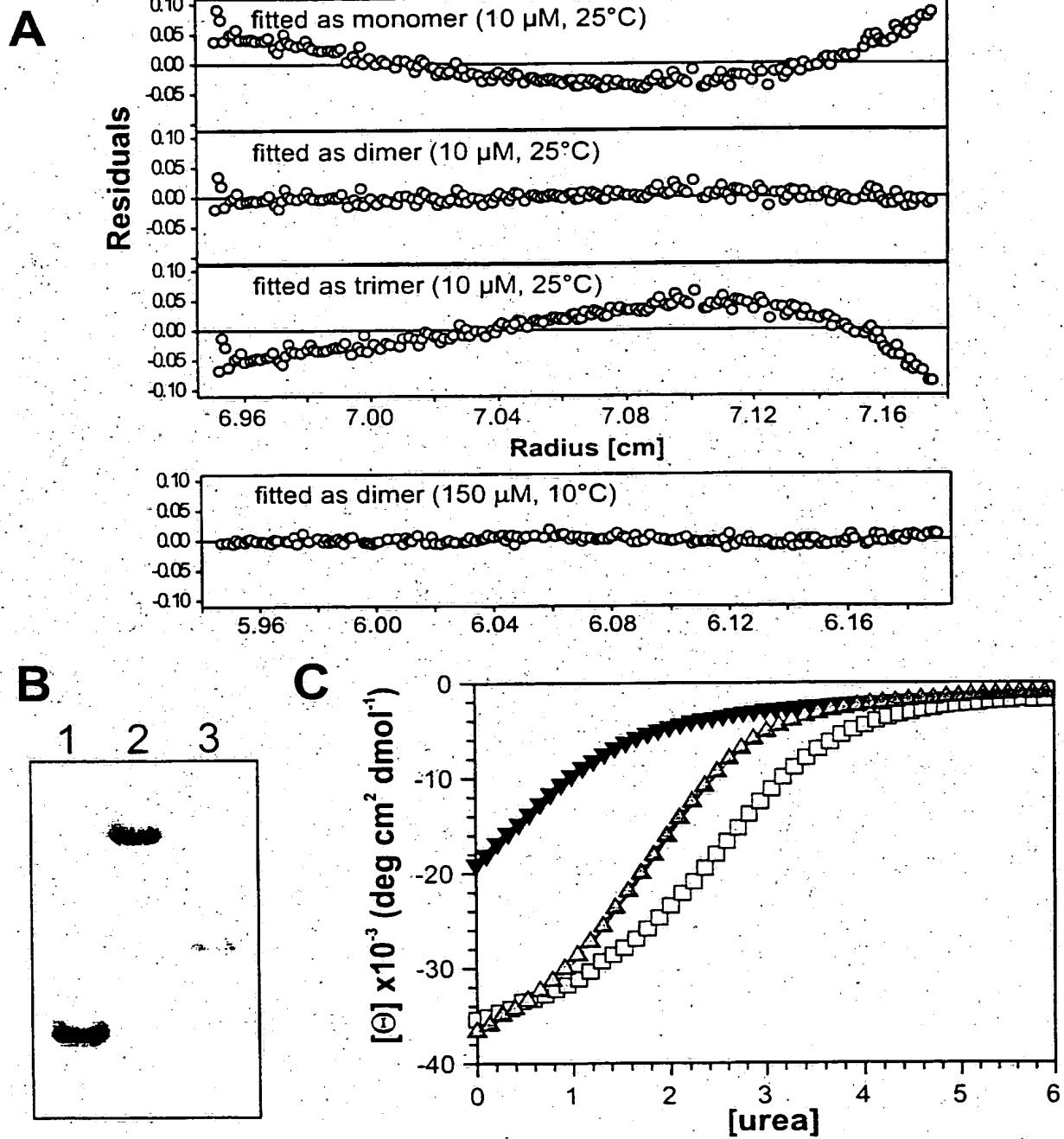
Figure 7:

Figure 8:

Figure 9:
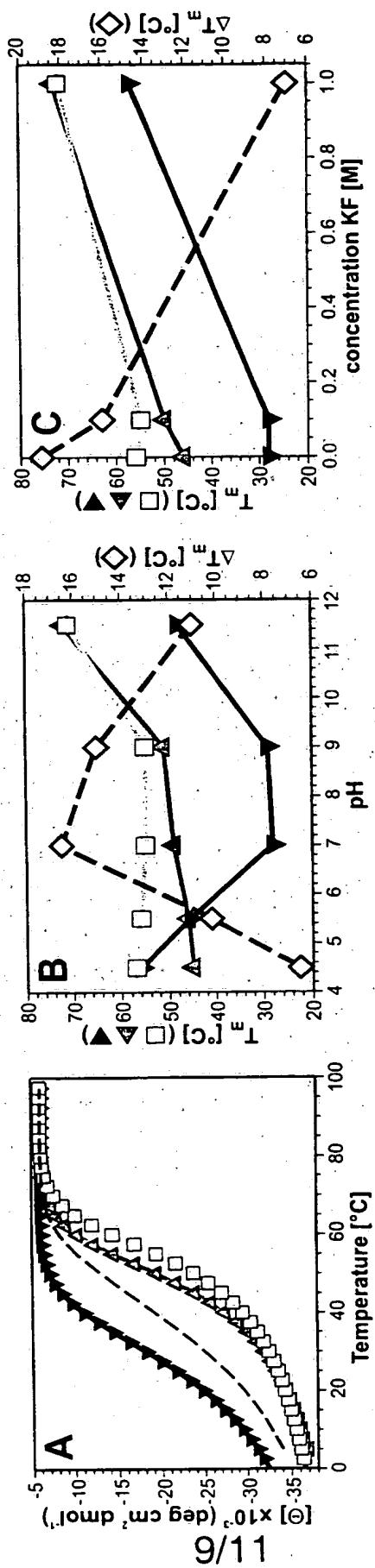
Figure 10:
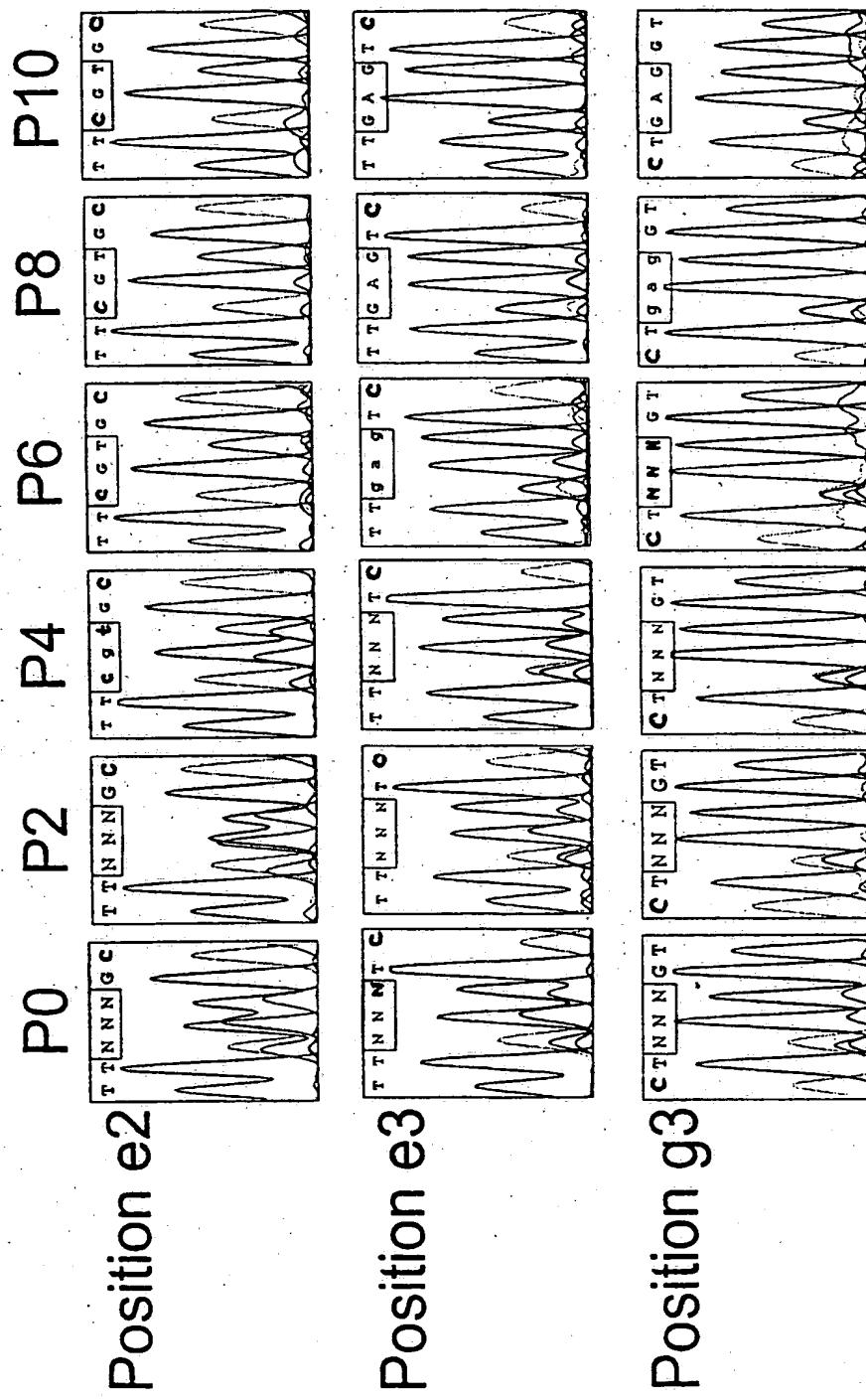
Figure 11:

(A)

|	
WinZipA1:	VAQLEEKVKTLRAQNYELKSRVQRLREQVAQL
WinZipA2:	VAQLRERVKTLRAQNYELESEVQRLREQVAQL
WinZipA3:	VAQLQEKVKTLRARNYELKSEVQRLEEKVAQL
WinZipA4:	VAQLEEQVKTLQARNYELKSKVQRLKEKVAQL
WinZipA5:	VAQLEERVKTLRAQNYELKSKVQRLEEQVAQL
WinZipA6:	VAQLEEQVKTLEAENYELKSKVQRLRERVAQL
WinZipA7:	VAQLQEQVKTLEAQNYELESEVQRLKEQVAQL
WinZipA8:	VAQLEERVKTLKAENYELESEVQRLKERVAQL
WinZipA9:	VAQLEEKVKTLKAKNYELKSKVQRLKEKVAQL
WinZipA10:	VAQLQEEVKTLQAENYELRSEVQRLEEEVAQL
WinZipA11:	VAQLRERVKTLRARNYELQSKVQRLKERVAQL

(B)

WinZipB1:	VDELQAEVDQLQDENYALKTKVAQLRKKVEKL
WinZipB2:	VDELKAEVDQLQDQNYALRTKVAQLRKEVEKL
WinZipB3:	VDELEAEVDQLKDQNYALKTKVAQLQKQVEKL
WinZipB4:	VDELRAKVDQLQDENYALETEVAQLQKRVEKL
WinZipB5:	VDELEAEVDQLEDQNYALQTRVAQLEKRVEKL
WinZipB6:	VDELKAKVDQLKDKNYALRTKVAQLRKKVEKL
WinZipB7:	VDELRAQVDQLQDKNYALRTRVAQLKKRVEKL
WinZipB8:	VDELQAEVDQLQDQNYALRTQVAQLKKKVEKL
WinZipB9:	VDELRAQVDQLEDQNYALETQVAQLEKEVEKL
WinZipB10:	VDELQAKVDQLKDENYALQTKVAQLQKRVEKL
WinZipB11:	VDELRAEVDQLEDENYALRTRVAQLRKQVEKL